TEACHING AN OLD DRUG NEW TRICKS: RE-PROFILING OF NEVIRAPINE AS AN HDL MODULATOR

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À minha avó Armandina,
Ao meu marido, Ricardo.

“Alone we can do so little; together we can do so much.”

Helen Keller

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RESUMO

Nevirapina (NVP), um inibidor da transcriptase reversa não-nucleósido (ITRNN) de 1ª geração, tem sido utilizado no tratamento da infeção pelo VIH-1 por mais de 20 anos. Este fármaco é um dos antirretrovirais mais prescritos a nível mundial devido à sua eficácia, baixo custo e perfil lipídico favorável, particularmente em relação aos aumentos dos níveis de colesterol das lipoproteínas de alta densidade (HDL). De facto, o tratamento antirretroviral com NVP tem sido associado à redução de lesões ateroscleróticas em doentes infectados pelo VIH. É importante salientar que não existem fármacos comercialmente disponíveis que permitam um aumento efectivo dos níveis de colesterol das HDL ou uma melhoria da funcionalidade das partículas de HDL. Apesar das vantagens clínicas associadas à NVP, este fármaco tem sido também associado à reações hepatotóxicas e de sensibilidade cutânea potencialmente graves, que impedem a sua indicação em outras áreas terapêuticas. Contudo, desenvolvimentos recentes na nossa compreensão da toxicocinética da NVP elucidaram que estas reações adversas estão relacionadas com vias de biotransformação e bioactivação específicas. A nossa hipótese, que se encontra fortemente embasada na literatura, é que o perfil benéfico da NVP na modulação das HDL é devido a um metabolito da NVP e não ao fármaco. De forma a testar esta hipótese, o principal objectivo do trabalho de investigação foi compreender as relações entre o tratamento com NVP, a biotransformação do fármaco e a modulação da funcionalidade das HDL, de modo a gerar conhecimento útil que permita o desenho racional de um novo fármaco modulador das HDL, com um melhor perfil toxicológico que a NVP. Este trabalho encontra-se focado em dois componentes do proteoma das HDL essenciais à funcionalidade destas partículas: Apolipoproteína A1 (ApoA1), o precursor e principal componente proteico das HDL, e a enzima antioxidante paraoxonase-1 (PON-1).

Primeiramente, a nossa abordagem experimental baseou-se em dois estudos clínicos exploratórios conduzidos com doentes infectados pelo VIH, com um desenho prospectivo e transversal, respectivamente (Capítulo 1). Onze doentes infectados pelo VIH a iniciar terapêutica com NVP foram acompanhados prospectivamente por um período de até 20 semanas. No estudo transversal, foram incluídos um total de 146

doentes naïve e 186 doentes tratados com NVP (400 mg NVP/dia durante pelo menos 3 meses). Os end-points avaliados nestes estudos clínicos foram os níveis de colesterol das HDL, ApoA1, anticorpos anti-HDL e anti-ApoA1, atividades paraoxonase (POase), arilesterase (AREase) e lactonase (LACase) da enzima PON-1. O tratamento com NVP foi associado a níveis mais elevados de colesterol das HDL e ApoA1, maiores atividades da PON-1 e níveis mais reduzidos de anticorpos anti-HDL e anti-ApoA1, o que sugere uma melhor funcionalidade das HDL entre os doentes tratados com NVP. A modulação temporal da funcionalidade das HDL ao longo do tratamento com NVP evidenciou-se no estudo prospectivo. O evento observado mais precocemente foi o decréscimo dos anticorpos anti-HDL, enquanto que mudanças nos restantes end-points foram observadas mais tardiamente. No estudo transversal, mulheres tratadas com NVP constituíram o grupo de doentes com níveis mais elevados de colesterol das HDL e ApoA1. A análise das relações entre as concentrações plasmáticas de NVP e dos seus três principais metabolitos de fase I revelou que concentrações mais elevadas de NVP estão associadas a níveis mais elevados de 2-hidroxi-NVP (2-OH-NVP), 12-hidroxi-NVP (12-OH-NVP) e particularmente a níveis mais elevados de 3-hidroxi-NVP (3-OH-NVP). Este padrão de metabolitos poderá reflectir uma indução preferencial do CYP2B6 pela NVP, o que levaria à formação de 3-OH-NVP. Adicionalmente, proporções mais elevadas de 3-OH-NVP foram associadas a menores proporções de 2-OH-NVP e 12-OH-NVP. A proporção de 2-OH-NVP foi também fortemente associada a níveis mais baixos de anticorpos anti-HDL. Estas observações parecem consistentes com a activação do receptor constitutivo de androstano (CAR) pela NVP, enquanto que o efeito tardio na modulação da funcionalidade das HDL sugere a acumulação de um metabolito da NVP com perfil lipídico mais favorável.

De modo a esclarecer a contribuição individual de cada via de biotransformação da NVP na modulação da PON-1 e da ApoA1, foram realizados estudos *in vitro* com três modelos de hepatócitos. A NVP e os seus principais metabolitos de fase I, 2-OH-NVP e 12-OH-NVP, foram incubados em culturas primárias 2D e 3D de hepatócitos de rato e também em culturas 2D da linha celular HepG2. Comprovou-se que o modelo 3D de hepatócitos é o mais adequado para investigar os efeitos da NVP e dos seus metabolitos na PON-1 (Capítulo 2) e na ApoA1 (Capítulo 3). O metabolito 12-OH-NVP promoveu um aumento

das três actividades da PON-1, após um período de exposição de apenas 4 dias. Por outro lado, os aumentos dos níveis de ApoA1 ocorreram após 12 dias de exposição: a NVP induziu um aumento de 43 % enquanto o 2-OH-NVP induziu um aumento de 86 %. Estas evidências *in vitro* sugerem que a formação de 12-OH-NVP parece ser o principal factor responsável pelo aumento das actividades da PON-1 induzido pela NVP, enquanto que os efeitos tardios do 2-OH-NVP apontam para um efeito na ApoA1 devido a geração e a acumulação de um metabolito de fase II.

Estas descobertas preliminares são particularmente relevantes considerando a importância da PON-1 e da ApoA1 como alvos terapêuticos. Por exemplo, a PON-1 tem sido implicada na patogénese de diversas doenças relacionadas com o envelhecimento. Relativamente à ApoA1, o aumento desta proteína anti-aterogénica tem sido considerado com uma das abordagens mais promissoras com vista ao aumento dos níveis de colesterol das HDL e para melhoria da função das HDL. Para além da área cardiovascular, níveis reduzidos de ApoA1 têm sido descritos em muitos tipos de neoplasias. Adicionalmente, considerando o perfil dimórfico dependente do sexo associado à modulação da ApoA1 induzida pela NVP, observado no estudo clínico transversal, foi formulada a hipótese de que a NVP ou um análogo pudessem ter uma aplicação terapêutica em neoplasias que afectam particularmente as mulheres. Esta suposição levou-nos ao cancro do ovário, uma neoplasia especificamente feminina e com mau prognóstico, caracterizada por uma expressão de ApoA1 diminuída no tecido ovárico e por níveis reduzidos de ApoA1 no plasma. Por esta razão, foram investigados os efeitos da ApoA1 e de um péptido mimético da ApoA1 no fenótipo maligno de células de cancro do ovário (Capítulo 4). A ApoA1 e o péptido 4F foram ambos capazes de reduzir a viabilidade de linhas celulares de cancro do ovário. A ApoA1 reduziu a capacidade de invasão das células SKOV3, enquanto que o péptido mimético da ApoA1 provocou a supressão da via de sinalização do Akt. De acordo com este efeito na via do Akt, o péptido mimético da ApoA1 tornou as células de cancro do ovário mais sensíveis à cisplatina. Este efeito foi observado não só *in vitro* mas também no ensaio *in ovo* em membrana corioalantóica (CAM), um modelo mais relevante do ponto de vista biológico. Estes resultados corroboram o efeito anti-tumorigénico da ApoA1 e do péptido mimético da ApoA1.

Em conclusão, com este trabalho de investigação foi possível contribuir para uma melhor compreensão do papel da biotransformação da NVP na modulação da funcionalidade das partículas de HDL. Esta abordagem inovadora, guiada pelo conhecimento das vias de biotransformação da NVP, poderá contribuir para o desenvolvimento de novos fármacos cujo alvo terapêutico seja a HDL. Tais fármacos podem ajudar a ampliar os recursos terapêuticos para diversas patologias que estão relacionadas com uma baixa funcionalidade das HDL, da aterosclerose às neoplasias.

ABSTRACT

The 1st generation non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) has been used for over 20 years for the treatment of HIV-1 infection. This drug is one of the most prescribed antiretrovirals worldwide due to its efficacy, low cost and lipid-friendly properties, particularly regarding increases in high density lipoprotein (HDL)-cholesterol levels. In fact, NVP-based antiretroviral treatment has been associated with a reduction of atherosclerotic lesions in HIV-infected patients. Importantly, there are no drugs available to effectively increase HDL-cholesterol levels or to improve the functionality of HDL particles. Despite NVP significant clinical advantages, this drug has been associated with severe hepatotoxic reactions and skin rash, which prevents its reprofiling for other therapeutic areas. However, recent developments in our understanding of NVP toxicokinetics have elucidated that these adverse reactions are biotransformation-driven and related to specific bioactivation pathways. Our hypothesis strongly based on literature was that the HDL-friendly profile of NVP was due to a metabolite rather than NVP itself. To address our hypothesis the main objective of the current research work was to understand the relations between NVP treatment, drug biotransformation and NVP-induced modulation of HDL functionality, in order to gain useful insights for the rational design of a new HDL modulator, with a better safety profile than NVP. In this work, we have been focused in two components of HDL proteome critically involved in the functionality of HDL particles: Apolipoprotein A1 (ApoA1), the precursor and main protein component of HDL, and the antioxidant enzyme paraoxonase-1 (PON-1).

Our experimental approach was firstly based on two exploratory clinical studies, conducted with HIV-infected patients with a prospective and a cross-sectional design, respectively (Chapter 1). Eleven HIV-infected patients starting NVP-based treatment were prospectively followed for up to 20 weeks. In the cross-sectional study, a total of 146 naïve patients and 186 NVP-treated patients (400 mg NVP/day for at least 3 months) were included. The end-points evaluated in these clinical studies were HDL-cholesterol, ApoA1, anti-HDL and anti-ApoA1 antibodies, paraoxonase (POase), arylesterase (AREase) and lactonase (LACase) activities of the PON-1 enzyme. NVP treatment was

associated with higher levels of HDL-cholesterol and ApoA1, higher PON-1 activities and lower levels of anti-HDL and anti-ApoA1 antibodies, suggesting an overall better HDL functionality among NVP-treated patients. The temporal modulation of HDL functionality throughout NVP treatment was evident in the prospective study. The earliest event observed was the decrease in anti-HDL antibodies, while changes on the remaining end-points were later observed. In the cross-sectional analysis, women on NVP treatment was the group with higher levels of HDL-cholesterol and ApoA1. An analysis of the relations between NVP plasma concentrations and its three main phase I metabolites showed that higher NVP concentrations were associated with higher levels of 2-hydroxy-NVP (2-OH-NVP), 12-hydroxy-NVP (12-OH-NVP) and particularly with higher levels of 3-hydroxy-NVP (3-OH-NVP). This might reflect a preferential induction of CYP2B6 by NVP, which generates 3-OH-NVP. Also, higher proportions of 3-OH-NVP were associated with lower proportions of 2-OH-NVP and 12-OH-NVP. The proportion of 2-OH-NVP was also strongly associated with lower levels of anti-HDL antibodies. This metabolite profile seems to be consistent with NVP-induced activation of constitutive androstane receptor (CAR), and the late modulatory effects on HDL functionality suggest the accumulation of a NVP metabolite with more lipid-friendly properties than NVP.

In order to clarify the individual contribution of each NVP biotransformation pathway affecting the modulation of PON-1 and ApoA1, we performed *in vitro* studies employing three different hepatocyte models. NVP and its main phase I metabolites, 2-OH-NVP and 12-OH-NVP, were incubated in 2D and 3D primary cultures of rat hepatocytes and also in 2D HepG2 cultures. The 3D hepatocyte model has proved to be the most suitable to investigate the effects of NVP and its metabolites on PON-1 (Chapter 2) and ApoA1 (Chapter 3). The 12-OH-NVP metabolite was a booster for the three activities of PON-1, after a short exposure of 4 days. Increases of ApoA1 levels only occurred after 12 days of exposure: up to 43 % for NVP and up to 86 % for 2-OH-NVP incubation. This *in vitro* evidence suggests that the formation of 12-OH-NVP seems to be the main factor responsible for the increase of PON-1 activities induced by NVP exposure, while the delayed onset effects of 2-OH-NVP on ApoA1 points towards a boosting effect due to the generation and accumulation of a phase II metabolite.

These preliminary findings are particularly relevant considering the importance of PON-1 and ApoA1 as therapeutic targets. For instance, PON-1 has been implicated in the pathogenesis of several aging-related diseases. Relatively to ApoA1, the upregulation of this anti-atherogenic protein has been considered one of the most promising approaches to increase HDL-cholesterol levels and improve HDL function. Beyond the cardiovascular field, decreased levels of ApoA1 have been reported in many types of cancer. Furthermore, considering the sex-dependent dimorphic profile of NVP-induced ApoA1 modulation observed in the cross-sectional clinical study, we hypothesised that NVP or an analogue might have a therapeutic application in malignancies affecting particularly women. This prompted us towards ovarian cancer, a women-specific malignancy with poor prognosis characterised by decreased ApoA1 expression in the ovarian tissue and decreased serum levels of ApoA1. We investigated the effects of ApoA1 and an ApoA1 mimetic peptide on the malignant phenotype of ovarian cancer cells (Chapter 4). The full-length ApoA1 and the 4F ApoA1 mimetic were both able to decrease the viability of ovarian cancer cell lines. ApoA1 was able to decrease the invasiveness of the aggressive SKOV3 cancer cells, while the ApoA1 mimetic peptide strongly suppressed Akt signalling. Accordingly, the ApoA1 mimetic peptide was able to sensitise ovarian cancer cells to cisplatin. This sensitisation effect was observed both *in vitro* and in the biologically relevant *in ovo* chorioallantoic membrane (CAM) model. These results support the anti-tumorigenic effect of ApoA1 and ApoA1 mimetic peptides.

In conclusion, with the current work, we have contributed to a better understanding of the role of biotransformation in the NVP-induced modulation of HDL particle functionality. This innovative biotransformation-oriented approach might contribute for the development of new drugs targeting HDL levels and quality that can help to expand the therapeutic arsenal for several diseases, from atherosclerosis to cancer, associated with poor HDL functionality.

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ACRONYMS AND ABBREVIATIONS

ABC – ATP-binding cassette transporters

ABV – abacavir

AhR – aryl hydrocarbon receptor

ALKP – alkaline phosphatase

ALT – alanine aminotransferase

ANOVA – Analysis of variance

ApoA1 – apolipoprotein A1

APS – adenosine 5'-phosphosulfate

AREase – arylesterase activity

BET – bromodomain and extra-terminal proteins

BMI – body mass index

BSA – bovine serum albumin

CAM – chorioallantoic membrane

CAR – constitutive androstane receptor

cART – combined antiretroviral therapy

CETP – cholesteryl ester transfer protein

Cmax – maximum plasma concentration

cPLA2 – cytosolic phospholipase A2

CYP450 – cytochrome P450

DHEA – dehydroepiandrosterone

DMSO – dimethyl sulfoxide

ECOD – ethoxycoumarin O-deethylase

EFV – efavirenz

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

ERα – oestrogen receptor α

ERK1/2 – extracellular signal-regulated kinase 1/2

EROD – ethoxyresorufin-O-deethylase

FBS – foetal bovine serum

FDA – Food and Drug Administration

FNT – farnesyltransferase

FOX – forkhead box protein

FTC - emtricitabine

GFP – green fluorescent protein

GGT – gamma-glutamyltransferase

GPCRs – G-protein-coupled receptors

GSH – glutathione

GSTs – glutathione *S*-transferases

HDL – high density lipoprotein

HIF – hypoxia-inducible factor

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HPLC – high performance liquid chromatography

HRP – horseradish peroxidase

IQR – interquartile range

LACase – lactonase activity

LCAT – lecithin-cholesterol acyltransferase

LDH – lactate dehydrogenase

LDL – low density lipoprotein

LPA – lysophosphatidic acid

LRH-1 – liver receptor homolog-1

LXR – liver X receptor

MAPK – mitogen-activated protein kinases

MDA – malondialdehyde

MHC – major histocompatibility complex

MMP-9 – matrix metalloproteinase 9

NEAAs – non-essential amino acids

NNRTI – non-nucleoside reverse transcriptase inhibitor

NRTI – nucleoside reverse transcriptase inhibitor

NVP – nevirapine

p125FAK – p125 focal adhesion kinase

p130CAS – p130 Crk-associated substrate

PAPS – 3'-phosphoadenosine 5'-phosphosulfate

PBS – phosphate buffered saline

PCSK9 – proprotein convertase subtilisin/kexin type 9

PGR-A – isoform A of progesterone receptor

PI – protease inhibitor

PI3K – phosphatidylinositol 3-kinase

POase – paraoxonase activity

PON-1 – paraoxonase-1

PPAR α – peroxisome proliferator-activated receptor alpha

PPAR γ – peroxisome proliferator-activated receptor gamma

PVDF – polyvinylidene difluoride

PXR – pregnane X receptor

RTKs – receptors tyrosine kinases

SEM – standard error of the mean

SGLT2 – sodium-glucose co-transporter-2

SR-B1 – scavenger receptor class B type 1

STAT3 – signal transducer and activator of transcription 3

SULTs – sulfotransferases

TDF – tenofovir disoproxil fumarate

TLR – Toll-like receptor

UGTs – uridine-diphosphate-glucuronosyltransferases

VEGF – vascular endothelial growth factor

YAP – Yes-associated protein

2D – two-dimensional

2-OH-NVP – 2-hydroxy-nevirapine

3D – three-dimensional

3-OH-NVP – 3-hydroxy-nevirapine

3TC – lamivudine

4-COOH-NVP – 4-carboxy-nevirapine

5-HETEL – 5-hydroxy-eicosatetraenoic acid lactone

8-OH-NVP – 8-hydroxy-nevirapine

12-OH-NVP – 12-hydroxy-nevirapine

INTRODUCTION

1. Nevirapine re-profiling: from an anti-HIV drug to an HDL modulator – overview of the research hypothesis and key ideas

Several antiretroviral drugs with different mechanisms of action and therapeutic targets are commercially available for the treatment and prophylaxis of human immunodeficiency virus (HIV)-infection (Arts and Hazuda, 2012) and many other small molecules are currently under development (Lalezari et al., 2015; Hermann, 2016; Dlamini and Hull, 2017; González, 2017; Kang et al., 2017; Yi et al., 2017). The availability of antiretroviral drugs for treating HIV-infection and the introduction of a therapeutic strategy based on a combination of at least three antiretrovirals, known as combined antiretroviral therapy (cART), had a tremendous impact on the outcome of this viral infection, which is now considered as a chronic manageable condition (Oversteegen et al., 2007; Barré-Sinoussi et al., 2013). According to the recommendations of the *World Health Organization* for treating HIV-infected adults and adolescents, the first line cART should consist of a nucleoside reverse transcriptase inhibitor (NRTI), such as emtricitabine (FTC) or lamivudine (3TC), in combination with tenofovir disoproxil fumarate (TDF) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), or alternatively, two NRTIs in combination with a NNRTI or an integrase inhibitor. Additionally, other classes of antiretroviral drugs can be included in a cART scheme as second or third line options, namely ritonavir-boosted protease inhibitors (PI) and integrase inhibitors (World Health Organization, 2016).

The current dissertation will be focused on the 1st generation NNRTI nevirapine (NVP) that received the approval of the U.S. *Food and Drug Administration* (FDA) over 20 years ago for the treatment of HIV-1 infection (Bowersox, 1996). This drug remains one of the most prescribed antiretrovirals, particularly in middle- and low-income countries (Oreagba et al., 2014; Dragovic et al., 2016), due to its positive cost-effectiveness evaluation (Maredza et al., 2013). In addition to its use for treating HIV infection, NVP is also commonly prescribed to pregnant women, newborns and breastfeeding babies of HIV-infected mothers to prevent vertical transmission of HIV (Maredza et al., 2013; Stevens and Lyall, 2014; World Health Organization, 2016; Lau et al., 2017). Furthermore, the availability of an extended-release formulation of NVP, allows a more convenient once-daily dosing (Ward and Slim, 2013). Also, the low incidence of adverse

reactions on the central nervous system (contrarily to the other 1st line NNRTI, efavirenz (EFV)) allows NVP prescription to HIV-infected patients with neuropsychiatric conditions or who are illicit drugs users (Medrano et al., 2008; Mbuagbaw et al., 2016). These factors have guaranteed NVP-based cART a prominent role in HIV-1 treatment strategies. Besides the above-mentioned clinical advantages, NVP has been also associated with beneficial changes in the lipid profile of HIV-infected patients, particularly regarding the high density lipoprotein (HDL). In fact, an association of NVP treatment with a better lipid profile and higher HDL-cholesterol levels has been demonstrated in patients on first cART (Clotet et al., 2003; van Leth et al., 2004), in experienced patients switching from a PI- to a NVP-based cART (Ruiz et al., 2001; Clotet et al., 2003), in pregnant HIV-infected women (Floridia et al., 2009) and also in uninfected newborns on NVP for HIV infection prophylaxis (Sankatsing et al., 2007).

HDL levels are strongly and independently associated with a decrease in cardiovascular disease risk (Gordon et al., 1989; The Emerging Risk Factors Collaboration, 2009). The HDL particle is, indeed, a crucial player in essential antiatherogenic processes, namely in reverse cholesterol transport (Kingwell et al., 2014) but also in many other physiological functions, such as regulation of immune responses and inflammation (Yu et al., 2010; Camps et al., 2011; Liu et al., 2015), modulation of male and female fertility (Miettinen et al., 2001; Bogan and Hennebold, 2010; Agarwal et al., 2015) and prevention of tumorigenesis (Zamanian-Daryoush et al., 2013). Despite the importance of measuring the levels of cholesterol contained in HDL particles, in recent years it has been increasingly recognised the therapeutic relevance of improving HDL functionality rather than merely increasing HDL levels (Rosenson et al., 2015; Rhee et al., 2017).

Despite intensive efforts to develop an HDL booster, to date there are no drugs to effectively improve HDL function and increase HDL levels (Table 1; Barter et al., 2007; Nissen et al., 2007; The AIM-HIGH Investigators, 2011; The HPS2-THRIVE Collaborative Group, 2014; Ferreira and Marques da Silva, 2016; Nicholls et al., 2016; Eyvazian and Frishman, 2017; Fadini et al., 2017). For instance, the strategy of inhibiting cholesteryl ester transfer protein (CETP) to block cholesteryl esters-depletion and concomitant triglyceride-enrichment of HDL particles, became one of the greatest failures in the search for an HDL booster ("Learning lessons from Pfizer's $800 million failure," 2011; Eyvazian and Frishman, 2017). In fact, Pfizer's torcetrapib was associated with

hyperaldosteronism, hypertension, increased risk of major cardiovascular events and death for any cause (Barter et al., 2007), while Lilly's evacetrapib has demonstrated no clinical benefit in the prevention of coronary events or cardiovascular death (Nicholls et al., 2011a; Eyvazian and Frishman, 2017), despite remarkable increases in HDL-cholesterol levels with both drugs. Another interesting case in this search for an HDL booster has been niacin. This drug has failed to demonstrate any benefit in the prevention of major coronary events, myocardial infarction or stroke despite significant increases in HDL-cholesterol levels (The AIM-HIGH Investigators, 2011; The HPS2-THRIVE Collaborative Group, 2014), being also associated with nonspecific toxicity (The HPS2-THRIVE Collaborative Group, 2014). The lack of clinical benefit with niacin can be explained by the generation of dysfunctional HDL particles (Batuca et al., 2016), which further supports the importance of modulating HDL function rather than merely increasing HDL levels. Notably, the increases in HDL achieved with NVP treatment (van der Valk et al., 2001; Clotet et al., 2003; van Leth et al., 2004; Sankatsing et al., 2007; Franssen et al., 2009; Podzamczer et al., 2011, 2014; Strehlau et al., 2012) are far more significant than those reported with peroxisome proliferator-activated receptor alpha (PPAR α) agonists (Nissen et al., 2007; Davidson et al., 2014; Ishibashi et al., 2016; Ferri et al., 2017), with statins in combination with ezetimibe (Ballantyne, 2003; Catapano et al., 2005; Ferreira and Marques da Silva, 2016) and with other therapeutic approaches such as the inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) with the monoclonal antibody evolocumab (Sullivan et al., 2012) or the inhibition of sodium-glucose co-transporter-2 (SGLT2) with dapagliflozin (Fadini et al., 2017). In fact, unlike NVP (Tohyama et al., 2009), the use of dapagliflozin was associated with decreased HDL function, measured by decreased cholesterol efflux capacity (Fadini et al., 2017). It is also interesting to note that NVP promotes higher increases in the levels of apolipoprotein A1 (ApoA1) than RVX-208 (Franssen et al., 2009; Nicholls et al., 2011b, 2016), a drug specifically designed to epigenetically modulate ApoA1 transcription through specific inhibition of second bromodomains in the bromodomain and extra-terminal (BET) proteins (Picaud et al., 2013). The upregulation of ApoA1, the precursor and main protein constituent of HDL particles, has been recently recognised as one of the most promising approaches to increase HDL levels and function (Gadkar et al., 2016). Besides, NVP ability to modulate HDL and ApoA1 is intrinsic and not dependent on HIV

replication control, as demonstrated by Sankatsing (2007) in uninfected newborns receiving NVP. Moreover, unlike other therapeutic approaches associated with no clinical benefits (Barter et al., 2007; Nicholls et al., 2011b, 2011a, 2016; The AIM-HIGH Investigators, 2011; The HPS2-THRIVE Collaborative Group, 2014; Eyvazian and Frishman, 2017; Fadini et al., 2017), NVP treatment was associated with a reduction of atherosclerotic lesions, assessed by carotid intima-media thickness measurements (Maggi et al., 2011; Gleason et al., 2016). Therefore, NVP seems to be an interesting starting point for the development of an HDL booster with significant clinical benefits. Despite of NVP association with severe toxic reactions (Pollard et al., 1998; Cattelan et al., 1999; Taiwo, 2006), recent developments in our understanding of NVP pharmacology and toxicology have clarified that these adverse reactions are biotransformation-driven, related to particular bioactivation pathways and caused by the formation of specific reactive species (Antunes et al., 2008, 2010a, 2010b; Chen et al., 2008; Caixas et al., 2012; Marinho et al., 2014b). These new insights have led us to the hypothesis that a metabolite or a derivative of NVP, with less potential for toxicity, can be reprofiled as an HDL booster, in order to fill the gap that still exists in current therapeutics for improving levels and quality of HDL particles.

The key ideas on the basis of the present research work can be summarised as follows:

- NVP is a 1st line antiretroviral drug (World Health Organization, 2016) associated with increases in HDL levels and an overall positive lipid profile in HIV-infected patients (van der Valk et al., 2001; Clotet et al., 2003; van Leth et al., 2004; Sankatsing et al., 2007; Franssen et al., 2009; Podzamczer et al., 2011, 2014; Strehlau et al., 2012);
- This HDL modulator effect is intrinsic to NVP and not related to the suppression of HIV replication (Sankatsing et al., 2007; Franssen et al., 2009);
- There are currently no drugs to effectively increase HDL levels or improve HDL function, and all the attempts to develop an HDL booster molecule have failed due to lack of clinical benefits or drug-related toxicity (Table 1);
- The HDL increases achieved with NVP treatment are far more significant than the increases reported with HDL-targeted therapies (Table 1), and the clinical

benefits in terms of atherosclerosis progression with NVP treatment are already demonstrated (Maggi et al., 2011; Gleason et al., 2016);

- The main drawback of NVP treatment is NVP-related toxicity, which is dependent on drug biotransformation and bioactivation through specific metabolic pathways (Antunes et al., 2010a; Caixas et al., 2012; Marinho et al., 2014c; Pinheiro et al., 2017).

The main goal of the research work described in the current dissertation was to clarify the relationship among NVP, its biotranformation and NVP-induced modulation of HDL levels and functionality. Using a strategy including *in vitro* and a prospective and cross-sectional clinical studies, we aimed to provide a rationale for the design of a clinically relevant and safer HDL booster molecule. A drug able to improve the quality of HDL particles would have applications not only in the field of cardiovascular diseases. For example, in recent years HDL components, such as ApoA1 (Zamanian-Daryoush and DiDonato, 2015) and the antioxidant enzyme paraoxonase-1 (PON-1) (Zhang et al., 2015), have emerged as interesting therapeutic targets in oncology, particularly for gynaecological malignancies (Su et al., 2010). Therefore, research in the modulation of HDL levels and quality has many potential translational applications and can bring real benefits to several patients suffering from a wider range of pathologies.

Table 1 Increase on high density lipoprotein-cholesterol and apolipoprotein A1 levels associated with different pharmacological interventions compared to nevirapine. *These pharmacological interventions, evaluated in prospective randomized clinical trials, were aimed at preventing cardiovascular events and atherosclerosis.*

Pharmacological interventions		***Dose***	***Treatment period***	***Mean % changes from baseline***		***Observations (eg. study design, safety concerns, clinical benefits)***	***References***
				HDL-cholesterol	***ApoA1***		
PPAR α Agonists	*Pemafibrate (K-877)*	0.2 mg twice a day	12 weeks	+ 21% *	+ 9% *		Ishibashi et al., 2016
	Fenofibrate	100 mg/day	12 weeks	+ 14% *	+ 6% *		
	LY-518674	25 µg/day	12 weeks	+ 16% *	+ 10% *	Up to 20% increase in LDL; Nephrotoxicity. [1]	Nissen et al., 2007
	Fenofibrate	200 mg/day	12 weeks	+ 14% *	+ 9% *	Nephrotoxicity. [1]	
Niacin	Extended-release niacin + laropiprant [2]	Niacin 2 g + laropiprant 40 mg	4 years	+ 14%	Non-quantified	Lack of clinical benefit against placebo; [3] toxicity. [4]	The HPS2 - THRIVE Collaborative Group, 2014
BET protein inhibitor	RVX-208 [5]	150 mg twice a day	12 weeks	+ 8% *	+ 6% *	Hepatotoxicity. [6]	Nicholls et al., 2011b

[1] Nephrotoxicity was related with marked increases in serum creatinine, above the upper limit of normal, in approximately 40% of patients treated with LY-518674 or with 200 mg fenofibrate.
[2] Laropiprant is a prostaglandin D_2 receptor 1 antagonist, which is prescribed in combination with niacin for reducing niacin-related flushing.
[3] Lack of clinical benefit against placebo, in terms of prevention of major coronary events, stroke or need for revascularization procedure, despite increases in HDL levels.
[4] Glucose homeostasis disturbances, serious adverse events associated with gastrointestinal and musculoskeletal systems, skin toxicity, increased incidence of infections and bleeding episodes.
[5] RVX-208 is a bromodomain and extra-terminal (BET) protein inhibitor aimed to increase the hepatic synthesis of ApoA1, based on an epigenetic therapeutic approach.
[6] Hepatotoxicity was related with increases in alanine aminotransferases and/or aspartate aminotransferases above 3 times the upper limit of normal, compared with placebo.
* HDL and ApoA1 levels differ from placebo and differ from baseline ($p < 0.05$).

Pharmacological interventions		*Dose*	*Treatment period*	*Mean % changes from baseline*		*Observations (eg. study design, safety concerns, clinical benefits)*	*References*
				HDL-cholesterol	*ApoA1*		
Statins monotherapy or combination therapy	*Rosuvastatin*	40 mg/day	Between 6 and 12 weeks	+ 10%	+ 6%	Pooled data from 14 clinical trials.	Catapano et al., 2005
	Ezetimibe [7] *+ simvastatin*	Ezetimibe 10 mg/day + simvastatin 20 mg/day	Between 5 and 12 weeks	+ 9%	+ 5%		
	Atorvastatin	Atorvastatin 20 mg/day	12 weeks	+ 4%	0%		Ballantyne et al., 2003
	Ezetimibe + atorvastatin	Ezetimibe 10 mg/day + atorvastatin 20 mg/day	12 weeks	+ 9% §	0%		
	Atorvastatin	40 mg/day	2 years	+ 3%	+ 3%	Nephrotoxicity. [8]	Davidson et al., 2014
	Fenofibric acid + atorvastatin	Fenofibric acid 135 mg/day + atorvastatin 40 mg/day	2 years	+ 7% §	+ 5%		
	Simvastatin	40 mg/day	3 years	+ 12%	+ 3%	Lack of clinical benefit. [9]	The AIM - HIGH Investigators, 2011
	Extended-release niacin + simvastatin	Niacin 2 g/day + simvastatin 40 mg/day	3 years	+ 25% §	+ 7%		

[7] Ezetimibe is a selective cholesterol-absorption inhibitor.

[8] Nephrotoxicity was related with increases in serum creatinine levels and decreases in estimated glomerular filtration rate among patients in the fenofibric acid plus atorvastatin arm.

[9] There was no advantage of adding niacin to simvastatin in terms of primary endpoints (*i.e.* death from coronary heart disease, myocardial infarction, ischemic stroke, acute coronary disease or need for coronary/cerebral revascularization), despite increases in HDL levels.

§ HDL levels differ between monotherapy and combination therapy ($p < 0.05$).

Pharmacological interventions		*Dose*	*Treatment period*	*Mean % changes from baseline*		*Observations (eg. study design, safety concerns, clinical benefits)*	*References*
				HDL-cholesterol	*ApoA1*		
Statins monotherapy or combination therapy (cont.)	Atorvastatin or rosuvastatin	Atorvastatin 10-40 mg/day or rosuvastatin 5-20 mg/day	26 weeks	+ 9% *	+ 11% *		Nicholls et al., 2016
	Atorvastatin or rosuvastatin + RVX-208	Atorvastatin 10-40 mg/day or rosuvastatin 5-20 mg/day + RVX-208 100 mg twice/day	26 weeks	+ 11% *	+ 13% *	Lack of clinical benefit; [10] hepatotoxicity. [11]	
	Atorvastatin	Atorvastatin optimal dose [12]	1 year	+ 1%	Non-quantified	Torcetrapib-related serious adverse events and increased risk of death. [14]	Barter et al., 2007
	Atorvastatin + torcetrapib [13]	Atorvastatin optimal dose [12] + torcetrapib 60 mg/day	1 year	+ 70% §			
	Rosuvastatin	10 mg/day	12 weeks	+ 6%	Non-quantified	Evacetrapib-related hypertension; no clinical benefit. [15]	Nicholls et al., 2011a; Eyvazian and Frishman, 2017.
	Rosuvastatin + evacetrapib [13]	Rosuvastatin 10 mg/day + evacetrapib 100 mg/day	12 weeks	+ 94% §			

[10] No advantage of adding RVX-208 to statins in terms of atherosclerotic plaque regression.
[11] Hepatotoxicity was related with increases in alanine aminotransferases and/or aspartate aminotransferases above 3 times the upper limit of normal, among patients in the RVX-208 arm.
[12] Patients received optimal dose of atorvastatin to achieve LDL-cholesterol levels lower than 100 mg/dL.
[13] Torcetrapib and evacetrapib are cholesteryl ester transfer protein (CETP) inhibitors.
[14] Torcetrapib was associated with higher systolic and diastolic blood pressure, disturbances in serum electrolytes, increased serum aldosterone, prolongation of QT interval and increased risk of stroke, transient ischemic attack, hospitalisation for unstable angina, increased risk of major coronary events and increased risk of death for any cause.
[15] No clinical benefits in terms of cardiovascular death, myocardial infarction, stroke, need for coronary revascularisation or unstable angina hospitalisation. § HDL levels differ between monotherapy and combination therapy ($p < 0.05$).

Pharmacological interventions		*Dose*	*Treatment period*	*Mean % changes from baseline*		*Observations (eg. study design, safety concerns, clinical benefits)*	*References*
				HDL-cholesterol	*ApoA1*		
SGLT2 inhibitors	Dapagliflozin	10 mg/day	12 weeks	0%	Non-quantified	Decreased HDL function. [16]	Fadini et al., 2017
PCSK9 inhibitors	Evolocumab [17]	420 mg every 4 weeks.	12 weeks	+ 7% *	+ 8% *		Sullivan et al., 2012
	Evolocumab + ezetimibe	Evolocumab 420 mg every 4 weeks + ezetimibe 10 mg/day	12 weeks	+ 12% *	+ 8% *		
NVP-based cART		Standard dosing schedules for treating/ preventing HIV infection.	24 weeks	+ 49% *¶	+ 19% *¶	Naïve patients	van der Valk et al., 2001;Clotet et al., 2003.
			48 weeks	+ 28% *¶	+ 18% *¶	Naïve patients	Podzamczer et al., 2011
			48 weeks	+ 43% ¶	Non-quantified	Naïve patients	van Leth et al., 2004
			96 weeks	+ 42% *	Non-quantified	Naïve patients	Podzamczer et al., 2014
			24 weeks	+ 16% *	+ 17% *	Experienced patients [18]	Franssen et al., 2009
			24 weeks	+ 20% *	Non-quantified	PI-experienced patients	Clotet et al., 2003
			2.5 years	+ 35% ¶	Non-quantified	PI-experienced children	Strehlau et al., 2012
			6 weeks	+ 8% ¶	+ 17% ¶	Uninfected newborns	Sankatsing et al., 2007

[16] Cholesterol efflux capacity was significantly lower *versus* placebo and *versus* baseline.

[17] Evolocumab is a monoclonal antibody targeting proprotein convertase subtilisin/kexin type 9 (PCSK9), the enzyme that mediates the trafficking of LDL receptor. This monoclonal antibody is particularly useful for statin-intolerant patients; it is administered subcutaneously.

[18] Patients (n=12) did not switch to NVP-based cART; NVP was added to current cART (zidovudine + lamivudine + abacavir). * HDL and ApoA1 levels differ from baseline ($p< 0.05$) ¶ HDL and ApoA1 levels differ between cART schemes ($p< 0.05$).

Abbreviations: ApoA1, apolipoprotein A1; BET, bromodomain and extra-terminal protein; cART, combined antiretroviral therapy; HDL, high density lipoprotein; HIV, human immunodeficiency virus; LDL, low density lipoprotein; NVP, nevirapine; PCSK9, proprotein convertase subtilisin/kexin type 9; PPAR α, peroxisome proliferator-activated receptor alpha; SGLT2, sodium-glucose co-transporter-2.

2. High density lipoprotein particles – functional importance of HDL proteome

HDL particles (Figure 1) are protein-rich lipoprotein complexes characterised by plurimolecular structure and heterogeneous function (Kontush et al., 2015). The HDL composition in terms of lipids and proteins is highly dynamic and varies largely during HDL metabolism, remodelling and assembling within bloodstream (German et al., 2006). A mature HDL particle is typically composed of a hydrophobic core containing cholesteryl esters and triglycerides, which is surrounded by an outer layer of amphipathic lipids (eg. phospholipids and sphingolipids), unesterified cholesterol, apolipoproteins, mainly ApoA1 and apolipoprotein A2 (ApoA2), enzymes and proteins that are essential for HDL metabolism and functionality (Rohrer et al., 2004; Kontush et al., 2015; Kowalska et al., 2015; Rysz-Górzyńska and Banach, 2016).

HDL particles have been classified in different subfractions according to a variety of criteria, such as protein/lipid content, density, size, shape, electrophoretic mobility, or even according to a combination of different criteria. For instance, HDL3c is a protein-rich subfraction characterised by a higher density and a smaller size while HDL2b is a lipid-richer fraction characterised by lower density and higher size; HDL fractions can also be classified as α-HDL, which corresponds to cholesterol ester-containing particles or preβ-HDL, which corresponds to lipid-poor particles (Table 2; Camont et al., 2011; Kontush et al., 2015; Rysz-Górzyńska and Banach, 2016). Conflicting observations have arisen regarding the atheroprotective properties associated with each subfraction of HDL (Maeda et al., 2012; Camont et al., 2013; Kasko et al., 2014; Tian et al., 2014; Joshi et al., 2016). Maeda (2012) reported the inverse correlation between the larger, less dense and lipid-enriched HDL2 subfraction and several parameters such as body mass index (BMI), glucose levels, insulinemia and carotid intima-media thickness. Concordantly, it was reported a switch towards smaller, lipid-poor HDL fractions, in patients with acute coronary syndrome (Tian et al., 2014) and arterial disease (Kasko et al., 2014). However, the smaller, denser and protein-enriched HDL3 subfraction was associated with an overall better HDL functionality (Camont et al., 2013) and recent analysis, conducted with large cohorts from the Jackson Heart Study and the Framingham Offspring Cohort Study, demonstrated that this HDL3 subfraction was associated with decreased incidence of coronary heart disease (Joshi et al., 2016).

Table 2 Characterisation of high density lipoprotein subfractions.

HDL subfraction		*Size (nm)*	*Density (g/mL)*	*Apolipoprotein content (mol/mol HDL)*		*Lipid content (%)*	*Shape*
				ApoA1	*Others*		
pre-β1-HDL		<7	<1.210	ApoA1-rich > 90%	–	Lipid-poor.	Discoidal
HDL 3	HDL 3c	7.2 – 7.7	1.167 – 1.200	2 – 3	≤ 1	Free cholesterol (8), cholesteryl esters (23), phospholipids (67) and triglycerides (2).	Spherical
	HDL 3b	7.7 – 8.2	1.147 – 1.167	3	1		
	HDL 3a	8.2 – 8.8	1.125 – 1.147	3 – 4	≤ 2		
HDL 2	HDL 2a	8.8 – 9.8	1.100 – 1.125	4	≤ 2	Free cholesterol (9), cholesteryl esters (36), phospholipids (52) and triglycerides (3).	Spherical
	HDL 2b	9.8 – 12.9	1.063 – 1.100	4 – 5	≤ 2		

The high density lipoprotein (HDL) subfractions are characterised according to their size, density, content of major HDL components and particle shape, as reported by Kunitake et al., (1985), Ishida et al., (1990), German et al., (2006), Kontush (2006) and McPherson et al., (2006). Apolipoprotein content for pre-β1-HDL is stated as a percentage of total particle content. Apolipoprotein content for HDL3 and HDL2 subfractions is stated as mol of apolipoprotein A1 (ApoA1) per mol of HDL (mol/mol HDL), or mol of other apolipoproteins per mol of HDL (mol/mol HDL). Lipid content is presented as a percentage of free cholesterol, cholesteryl esters, phospholipids and triglycerides on HDL3 and HDL2 subfractions. All data presented here is relative to HDL of healthy normolipidemic individuals.

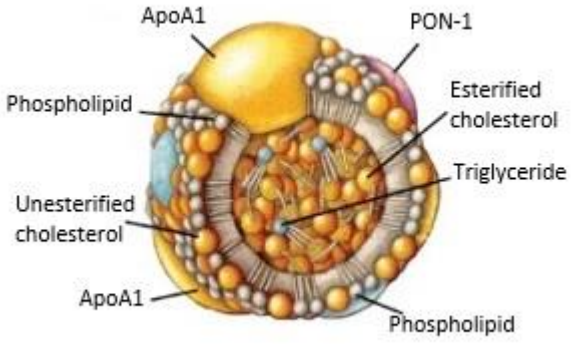

Figure 1 Composition of high density lipoprotein. *High density lipoprotein (HDL) particles are plurimolecular complexes (Kontush et al., 2015) which present a highly dynamic composition that varies during HDL metabolism (German et al., 2006). A mature HDL particle is composed of an outer layer of amphipathic lipids (mainly phospholipids), unesterified cholesterol and proteins, such as apolipoprotein A1 (ApoA1) or the antioxidant enzyme paraoxonase-1 (PON-1). The hydrophobic core contains esterified cholesterol and triglycerides (Rohrer et al., 2004; Kontush et al., 2015; Rysz-Górzyńska and Banach, 2016). Adapted from Forti and Diament, 2006. The original image is under a Creative Commons Attribution License (CC BY-NC).*

The above-mentioned difficulties in the identification of the most atheroprotective of HDL subfractions may stem from the complexity of HDL biology. In fact, HDL is highly heterogeneous and dynamic in composition, and both HDL lipidome (German et al., 2006; Camont et al., 2013) and proteome (Chistiakov et al., 2017; Rhee et al., 2017) can affect its biological activity at different extents. Noteworthily, differences in HDL quality or functionality are insufficiently explained by HDL-cholesterol levels or HDL subfractions (Dullaart et al., 2014). Therefore, simply measuring HDL-cholesterol or classifying HDL particles in different subfractions is probably not the most suitable approach for assessing the complex HDL biology. In fact, it has been increasingly recognised the importance of evaluating HDL function, in addition to the traditional assessment of lipid profile (Kontush et al., 2015; Rosenson et al., 2015; Rhee et al., 2017). Regarding HDL proteome components, ApoA1 is considered the major structural and functional protein, accounting for approximately 70% of total HDL protein composition (Kontush et al., 2015). In addition to ApoA1, the antioxidant enzyme PON-1 is also an essential player contributing for HDL antioxidant, anti-inflammatory and anti-atherosclerotic functions (Chistiakov et al., 2017). Thus, the following sections will focus on the functional properties of ApoA1 and PON-1 and how these proteins affect HDL functionality.

2.1. Apolipoprotein A1

2.1.1. Apolipoprotein A1 – functional role on reverse cholesterol transport and cholesterol homeostasis

As previously stated, ApoA1 is the main protein constituent of HDL particles and a key component for their functionality (Kingwell et al., 2014; Kontush et al., 2015). The pleiotropic effects of ApoA1 (Tuteja and Rader, 2014) substantiate the importance of this protein for several physiological functions, from fertility and reproduction (Ariel et al., 1994; Miettinen et al., 2001; Bogan and Hennebold, 2010; Agarwal et al., 2015) to the regulation of immune responses (Srinivas et al., 1991; Tada et al., 1993; Wilhelm et al., 2010; Yu et al., 2010; Zamanian-Daryoush et al., 2013). However, the best-known role of this apolipoprotein is in the maintenance of cholesterol homeostasis and in

promoting reverse cholesterol transport (Kingwell et al., 2014). In fact, reverse cholesterol transport (Figure 2) is an essential physiological process that will promote the decrease of the cellular cholesterol content on peripheral tissues and the subsequent transport of those lipids to the liver, for metabolism and excretion (Hassan et al., 2006; Heinecke, 2012). ApoA1 is mainly synthesized in the liver, small intestine and proximal colon (Kingwell et al., 2014; Gkouskou et al., 2016). After ApoA1 secretion into blood circulation and mesenteric lymph, this protein can associate with phospholipids and cholesterol to generate pre-β HDL particles. The ApoA1 interaction with phospholipids and cholesterol will be mediated by the hydrophobic C-terminal domain of this apolipoprotein (Nguyen et al., 2013). The initial lipid enrichment of pre-β HDL particles is mediated by ApoA1 interaction with ATP-binding cassette (ABC)A1 transporters. These ABC transporters will promote the efflux of phospholipids and unesterified cholesterol from peripheral tissues to HDL, being ApoA1 a very efficient lipid acceptor in this process (German et al., 2006; Vedhachalam et al., 2007; Kingwell et al., 2014; Mei and Atkinson, 2015). The plasmatic lecithin-cholesterol acyltransferase (LCAT) enzyme associates with discoidal HDL particles. ApoA1 acts as a co-factor for LCAT, which is responsible for catalysing the esterification of free cholesterol. This esterification reaction will induce the remodelling of HDL to spherical particles with a hydrophobic core of cholesteryl esters (Kunnen and Van Eck, 2012; Kingwell et al., 2014). These spherical HDL particles can be further loaded with unesterified cholesterol and phospholipids from peripheral cells via scavenger receptor class B type 1 (SR-B1)-mediated efflux or, alternatively, via ABCG1 transporter (Kennedy et al., 2005; Kingwell et al., 2014). However, since the ABCG1-mediated efflux is not exclusively directed towards HDL, other lipoproteins, such as the proatherogenic low density lipoprotein (LDL) and very low density lipoprotein particles can receive lipids via ABCG1 (Niesor, 2015). On the final steps of reverse cholesterol transport, ApoA1 promotes the uptake of HDL-cholesterol and cholesteryl esters by hepatocytes through interaction with SR-B1 (Yuhanna et al., 2001; Kingwell et al., 2014). Additionally, ApoA1 interaction with the β-chain of ATP synthase on the surface of hepatocytes can promote the uptake of the entire HDL particle (*holo*-HDL) by receptor-mediated endocytosis (Martinez et al., 2003; Kingwell et al., 2014). In the hepatic tissue, cholesterol is oxidised to bile acids – 3α-7α-12α-trihydroxy-5β-cholanoic acid (cholic acid) and 3α-7α-dihydroxy-5β-cholanoic acid

(chenodeoxycholic acid) – that are excreted into the bile after conjugation with glycine or taurine (Vaz and Ferdinandusse, 2017).

This brief description of the sequence of events during reverse cholesterol transport highlights the crucial functional role of ApoA1 in different steps of this process. ApoA1, derived from the liver or intestine, is an essential player in the interaction with amphipathic lipids (Nguyen et al., 2013), in the lipid enrichment of HDL particles (Vedhachalam et al., 2007; Mei and Atkinson, 2015) and also for cholesterol metabolism (Kunnen and Van Eck, 2012) and uptake of lipids by the liver (Yuhanna et al., 2001; Martinez et al., 2003). Moreover, ApoA1 is also expressed at significant levels in steroidogenic tissues, contributing locally to cholesterol homeostasis and consequently to the regulation of hormonal levels (Bogan and Hennebold, 2010). Considering its anti-inflammatory and anti-atherogenic properties (Nofer et al., 2002; Murphy et al., 2012; Bisoendial et al., 2015), the development of pharmacological strategies to promote ApoA1 upregulation is of utmost importance (Tuteja and Rader, 2014).

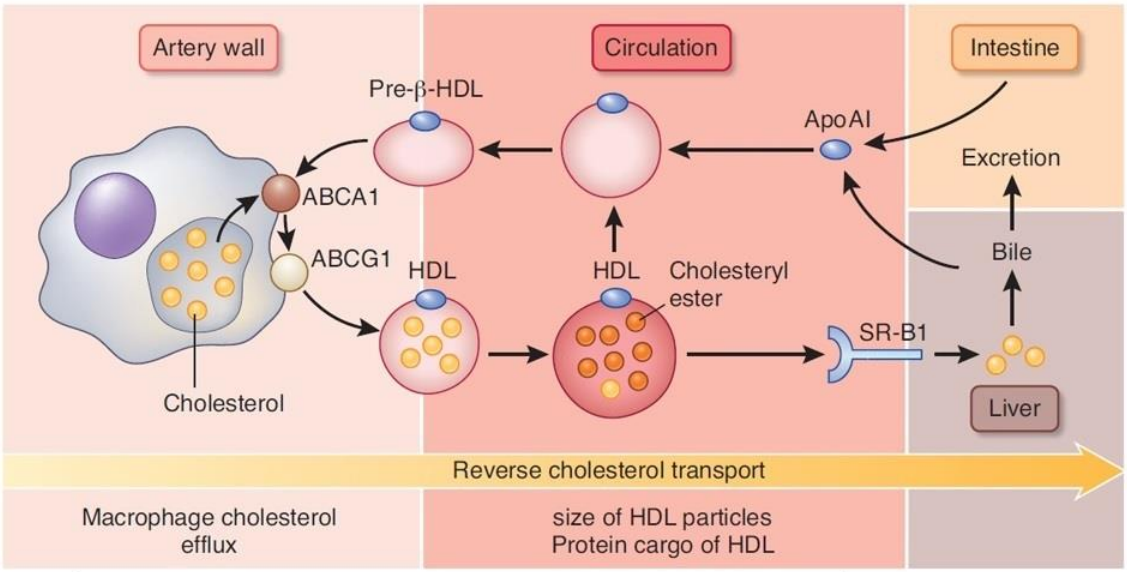

Figure 2 Overview of reverse cholesterol transport by high density lipoprotein. *Apolipoprotein A1 (ApoA1) is synthesized in the liver and intestine and subsequently secreted into circulation. Once in circulation, ApoA1 associates with phospholipids and cholesterol generating pre-β HDL particles (Kingwell et al., 2014). ApoA1 interaction with ATP-binding cassette transporters (ABCA1, ABCG1) mediates the lipid enrichment of HDL particles, allowing the efflux of phospholipids and unesterified cholesterol from peripheral tissues (eg. macrophages in the artery wall) to HDL (Kennedy et al., 2005; Vedhachalam et al., 2007). The lecithin-cholesterol acyltransferase (LCAT) enzyme associates with discoidal HDL particles, promoting the esterification of free cholesterol and inducing the remodelling of HDL to spherical particles (Kunnen and Van Eck, 2012). On the final steps of reverse cholesterol transport, the uptake of HDL-cholesterol and cholesteryl esters by hepatocytes occurs upon ApoA1 interaction with the scavenger receptor class B type 1 (SR-B1) (Yuhanna et al., 2001; Kingwell et al., 2014). In the liver, cholesterol is metabolised and excreted into bile (Vaz and Ferdinandusse, 2017). Adapted from Heinecke, 2012; with permission from the author, Prof Jay Heinecke.*

2.1.2. Physiological role of apolipoprotein A1 on steroidogenic tissues – the ovarian example

HDL and its main apolipoprotein are implicated in the regulation of several processes of fundamental importance for ovarian physiology. Early studies in this regard demonstrated that HDL-cholesterol concentration in follicular fluid of human ovaries is similar to plasma concentrations (Simpson et al., 1980), while ApoA1 levels in follicular fluid can reach approximately 60-75% of plasma concentration (Enk et al., 1986). In fact, HDL and ApoA1 can modulate ovarian physiology and female fertility in many ways (von Otte, 2005; Bogan and Hennebold, 2010; Becker et al., 2011). For instance, the interruption of progesterone production by the *corpus luteum* (*i.e.* luteal regression or functional luteolysis) is an essential event in non-conception cycles, allowing the beginning of the next menstrual period (Stouffer et al., 2013). As a steroidogenic tissue, the *corpus luteum* depends on reverse cholesterol transport for the regulation of its hormonal production, since cholesterol is a substrate for progesterone synthesis. The circulating ApoA1 might contribute to the cholesterol efflux at this tissue, however there is evidence of substantial ApoA1 synthesis in the ovary, and at particularly high levels in the *corpus luteum* throughout all the luteal phase (Bogan and Hennebold, 2010). Moreover, ApoA1 expression in ovarian granulosa cells is induced by the isoform A of progesterone receptor (PGR-A); in fact, the binding of progesterone to PGR-A induces dimerization of two progesterone-PGR-A complexes that interact with a hormone responsive element in the promoter region of target genes, mainly ApoA1 (Allan et al., 1992; Sriraman et al., 2010), further supporting the role of this apolipoprotein in the regulation of ovarian steroidogenesis (Figure 3). Besides ApoA1, many other key proteins involved in cholesterol homeostasis are significantly expressed in the ovary. A good example is the high-affinity HDL receptor SR-B1, whose expression in the ovary is approximately 25-fold higher than in liver (Landschulz et al., 1996). SR-B1 expression is further induced on progesterone-producing *corpus luteum* cells, in response to estrogen stimulation (Landschulz et al., 1996; Johnson et al., 1998; Wang et al., 2015), which supports the importance of HDL-cholesterol uptake for hormonal synthesis (Figure 3). Importantly, some SR-B1 polymorphisms were implicated in female infertility (Christianson and Yates, 2012). In addition to ApoA1 and SR-B1, the expression of ABCA1

and ABCG1 transporters in luteal cells (Bogan and Hennebold, 2010; Bogan et al., 2012), that is mechanistically mediated by liver X receptor (LXR) activation (Bogan et al., 2012), and the expression of LCAT enzyme (Bogan and Hennebold, 2010) by granulosa cells, further confirm the importance of cholesterol balance and cholesterol metabolism in ovarian physiology and female fertility (Kuokkanen et al., 2016).

In addition to the balance between cholesterol uptake and efflux, sulfonation of steroid hormones precursors is an essential mechanism for the regulation of steroidogenesis (Geyer et al., 2016). For instance, the sulfonated form of dehydroepiandrosterone (DHEA) is regarded as a biologically inactive steroid that can be converted into its active form in several steroidogenic tissues, upon hydrolysis mediated by steroids sulfatases. After this conversion, DHEA is available for estrogens and testosterone biosynthesis (Geese and Blanchard Raftogianis, 2001; Geyer et al., 2016). There are two isoforms of sulfotransferase (SULT)2B1, SULT2B1a and SULT2B1b, expressed at high levels in the ovary (Geese and Blanchard Raftogianis, 2001), which are able to promote the sulfonation of DHEA and other 3β-hydroxysteroids, therefore regulating the bioavailability of steroids hormones in this tissue (Geese and Blanchard Raftogianis, 2001; Falany and Rohn-Glowacki, 2013). Moreover, the SULT2B1b, unlike SULT2B1a, might also promote cholesterol sulfonation (Coughtrie, 2016). Interestingly, ApoA1 can stabilise SULT2B1b mRNA and protein levels in platelets, increasing its stability and activity towards cholesterol (Yanai et al., 2004). Thus, it is at least plausible that ApoA1 can also modulate SULT2B1 activity in the ovarian tissue (Figure 4), however this hypothesis has not yet been tested to date.

Angiogenesis is another essential process in ovary physiology. The formation of new capillary vessels takes place during the development of the ovarian follicle and also during its transition into the *corpus luteum* (Rizov et al., 2017). As referred before, the follicular fluid is extremely rich in HDL and ApoA1 (Simpson et al., 1980; Enk et al., 1986). However, it was reported that the ability of HDL particles to stimulate endothelial cell proliferation was not due to the ApoA1 fraction. Instead, this effect of HDL was attributed to its lipid fraction, probably through activation of protein kinase C (PKC) and phosphorylation of both extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase Akt (von Otte, 2005).

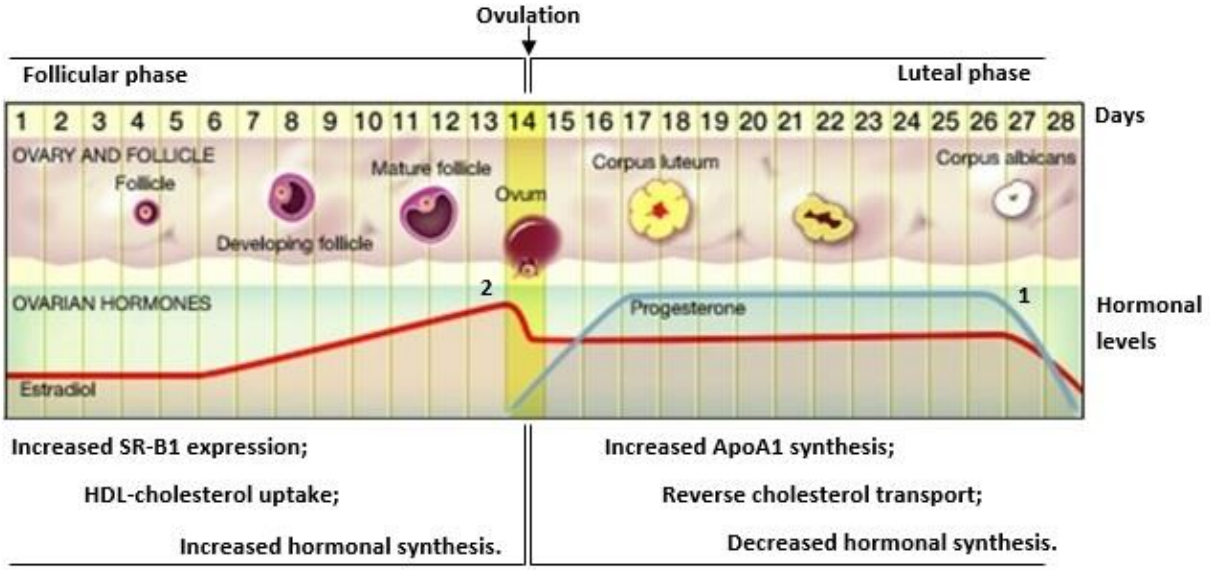

Figure 3 Interplay between high density lipoprotein-cholesterol, apolipoprotein A1 and ovarian steroidogenesis. *The luteal regression that occurs after ovulation involves interruption of progesterone production by the corpus luteum, causing a decrease in progesterone plasma levels (**1**; Stouffer et al., 2013). This process of functional luteolysis is dependent on reverse cholesterol transport. Apolipoprotein A1 (ApoA1) is synthesised in the ovary, particularly in the corpus luteum during all the luteal phase (Bogan and Hennebold, 2010). ApoA1 expression in ovarian granulosa cells is induced by progesterone binding to progesterone receptor A (PGR-A) (Sriraman et al., 2010). Contrarily, uptake of high density lipoprotein (HDL)-cholesterol is essential for hormonal synthesis. Therefore, the scavenger receptor class B type 1 (SR-B1), a high-affinity HDL receptor, is induced on progesterone-producing cells in the corpus luteum, upon estrogen stimulation (**2**), then promoting further HDL-cholesterol uptake for hormonal synthesis (Landschulz et al., 1996; Johnson et al., 1998; Wang et al., 2015). In addition, other genes involved in cholesterol homeostasis are induced in granulosa cells during the ovarian cycle (Bogan and Hennebold, 2010; Bogan et al., 2012), supporting the relevance of cholesterol balance for ovarian steroidogenesis. Adapted from Haimov-Kochman and Berger, 2014. The original image is under a Creative Commons Attribution License (CC BY).*

Taken together, expanding evidence indicates a key role for ApoA1 in ovarian physiology, particularly in the regulation of *de novo* steroidogenesis, through reverse cholesterol transport (Figure 3) and possibly in the regulation of steroids biosynthesis through stabilisation of SULTs (Figure 4). In contrast, the HDL-driven stimulation of angiogenesis is an ApoA1-independent process (von Otte, 2005).

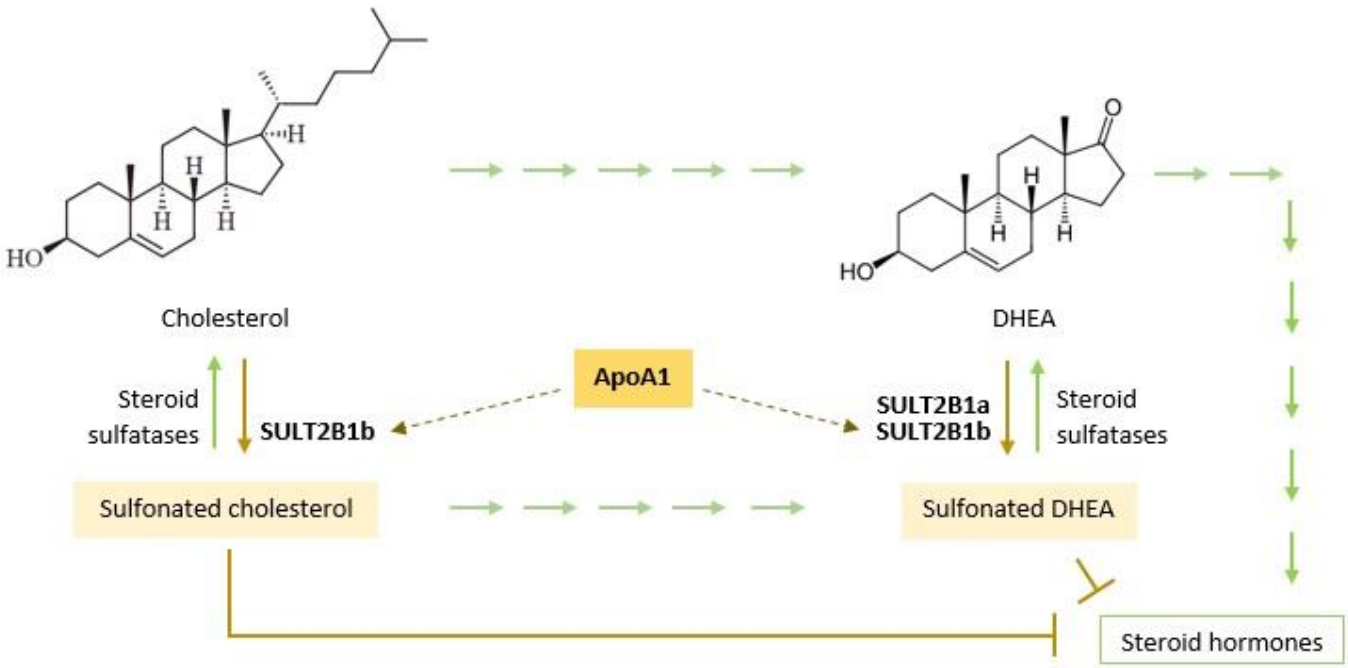

Figure 4 Interaction between apolipoprotein A1 and sulfotransferases might affect ovarian steroidogenesis. *Sulfotransferases (SULT)-mediated conjugation of steroids precursors represents an effective mechanism for regulating steroidogenesis, reducing hormonal synthesis. In fact, sulfonated forms of cholesterol and dehydroepiandrosterone (DHEA) do not undergo the biosynthetic pathways of steroid hormones. Thus, sulfonation (solid yellow arrows) contributes to the negative regulation of sex hormones synthesis (solid yellow lines); however, the sulfonated forms of steroids precursors can readily become available for steroidogenesis (solid green arrows) upon sulfatases-mediated hydrolysis (Geese and Blanchard Raftogianis, 2001; Geyer et al., 2016). Cholesterol is a substrate for SULT2B1b while DHEA can be metabolised by SULT2B1a or SULT2B1b (Geese and Blanchard Raftogianis, 2001). It is reported that apolipoprotein A1 (ApoA1) contributes, at least in platelets, for the stabilisation of SULT2B1b (yellow dashed line), increasing enzyme levels and activity towards steroids precursors (Yanai et al., 2004). Therefore, this interaction between ApoA1 and SULT2B1 might constitute another mechanism by which ApoA1 regulates ovarian steroidogenesis.*

2.1.3. Emerging role of apolipoprotein A1 as an anti-tumorigenic protein – the case of ovarian cancer

Ovarian cancer is the 7th most common neoplastic disease among women worldwide (Coburn et al., 2017). Up to 85% of patients with epithelial ovarian cancer who achieve full remission after 1st line chemotherapy will develop recurrent disease (Foley et al., 2013). Moreover, the effectiveness of surgery and radiotherapy remains largely unsatisfactory (Webber and Friedlander, 2017) and the 5-year survival rate is as low as 46% (Miller et al., 2016). For these reasons, the identification of new therapeutic targets

and the development of new therapies for ovarian cancer are urgently needed. Recently, ApoA1 has been included in a panel of 5 biomarkers that received FDA approval as a multiplex test for assessing the likelihood of malignancy of an ovarian mass (Nolen and Lokshin, 2013). The decision of including ApoA1 in this diagnostic test, along with the traditional cancer antigen 125 (CA125) and three other biomarkers, was based on the observation of remarkable ApoA1 decreases in serum and also in the ovarian tissue of patients with ovarian cancer (Clarke et al., 2011; Stavnes et al., 2014; Wegdam et al., 2014). Reduced levels of serum ApoA1 were also observed in several other types of tumors, including metastatic hepatocellular carcinoma (Qin et al., 2013), cholangiocarcinoma (Wang et al., 2009), gastric tumors (Chong et al., 2010), pancreatic cancer (Ehmann et al., 2007), colorectal cancer (Jung et al., 2015), breast cancer (Huang et al., 2006) and endometrial cancer (Takano et al., 2010; Rižner, 2016). Moreover, higher serum ApoA1 levels were associated with better prognosis and longer overall survival among patients suffering from renal cell cancer (Walter et al., 2012; Guo et al., 2016), non-metastatic nasopharyngeal carcinoma (Luo et al., 2015), advanced-stage non-small cell lung cancer (Cheng et al., 2015) and ovarian cancer (Stavnes et al., 2014). These associations between serum ApoA1 levels and several malignancies gave rise to the hypothesis that ApoA1 may have intrinsic anti-tumorigenic properties. In fact, increasing evidence supports that low levels of ApoA1 in cancer patients might be a causal factor in the development and progression of malignancies (Edelson, 2010; Su et al., 2010; Zamanian-Daryoush et al., 2013; Zamanian-Daryoush and DiDonato, 2015). The possible molecular mechanisms that might be underlying the anti-tumorigenic effect of ApoA1 are presented below, with special emphasis on ovarian cancer. These mechanisms are also summarised in Figure 5.

Binding of apolipoprotein A1 to lysophosphatidic acid

ApoA1 and ApoA1 mimetic peptides are able to bind to pro-inflammatory phospholipids and this is an important property contributing to ApoA1 and HDL anti-inflammatory and anti-atherogenic function (Van Lenten et al., 2009). It is known that some pro-inflammatory lysophospholipids can promote cell proliferation and cancer progression through the activation of several intracellular pathways (Xu et al., 1995; Fang et al.,

2002; Sako et al., 2006; Li et al., 2009; Jeon et al., 2010; Saunders et al., 2010; Liu et al., 2012; Ward and Dhanasekaran, 2012; Cai and Xu, 2013; Jeong et al., 2013; Riaz et al., 2016). An example of one of these pro-inflammatory lipids is lysophosphatidic acid (LPA), whose plasma levels are increased in approximately 90% of all ovarian cancer patients (Mills and Moolenaar, 2003; Bast et al., 2009). In fact, LPA can activate a variety of signalling pathways with importance in ovarian cancer progression (Figure 5). For instance, LPA induces tyrosine phosphorylation of p125 focal adhesion kinase (p125FAK) and activation of mitogen-activated protein kinases (MAPKs) (Xu et al., 1995). The activation of p125FAK can lead to increased ovarian cancer cell motility, invasion and metastasis (Sulzmaier et al., 2014), while activation of MAPKs has oncogenic potential through induction of ovarian cancer cells proliferation, survival and migration (Reddy et al., 2003; Smolle et al., 2013). LPA treatment also induced migration of mouse epithelial ovarian cancer ID8 cells, through phosphorylation of cytosolic phospholipase A2 (cPLA2) via MAPK/ERK signalling pathway (Li et al., 2009). Moreover, LPA stimulates tyrosine phosphorylation of p130 Crk-associated substrate (p130CAS), being the Gαi2 protein the intermediate in this pathway, which also contributes for LPA-mediated invasive phenotype and migration of ovarian cancer cells (Ward and Dhanasekaran, 2012). Furthermore, it was also demonstrated that LPA, upon binding to LPA receptor 3 (LPA3), induces dephosphorylation of Yes-associated protein (YAP), one of the major downstream effectors of the Hippo pathway. Dephosphorylation increases YAP nuclear translocation, enhancing invasion and migration of ovarian cancer cells (Cai and Xu, 2013). Additionally, LPA increases the expression of the transcriptional co-activator with PDZ-binding motif (TAZ) protein, another downstream effector of Hippo signalling pathway, further contributing for LPA-induced migration of ovarian cancer cells (Jeong et al., 2013).

Importantly, LPA stimulation, via Gαi/o βγ proteins coupled to LPA receptors, leads to activation of phosphatidylinositol 3-kinase (PI3K) resulting in Akt phosphorylation (Figure 5; Saunders et al., 2010; Riaz et al., 2016). Akt subsequently promotes phosphorylation of forkhead box protein (FOX)O3A, causing its proteasomal degradation and preventing expression of FOXO3A target genes (Huang and Tindall, 2011; Lam et al., 2013; Nestal de Moraes et al., 2016). While FOXO3A is regarded as a

tumour suppressor gene, FOXM1 is an oncogenic transcription factor (Gomes et al., 2013; Lam et al., 2013; Nestal de Moraes et al., 2016), which is induced in ovarian cancer cells upon LPA stimulation (Fan et al., 2015). Thus, LPA has the potential to promote degradation of FOXO3A and upregulation of FOXM1, driving the PI3K/Akt/FOXO3A/FOXM1 axis towards tumorigenesis and chemoresistance. In fact, the FOXM1 protein is crucially involved in the acquisition of resistance to platinum and also to other chemotherapeutic agents (Gomes et al., 2013; Lam et al., 2013; Zhao et al., 2014; Nestal de Moraes et al., 2016) that are administered as second-line and salvage therapy for ovarian cancer (Oronsky et al., 2017). In this regard, it is important to highlight that the emergence of resistance to chemotherapy is one of the biggest challenges in the management of ovarian cancer (Webber and Friedlander, 2017). Therefore, the development of new therapeutic strategies targeting Akt and FOX is of utmost importance in this field (Fraser et al., 2008; Peng et al., 2010; Lam et al., 2013; Zhao et al., 2014; Lokadasan et al., 2016; de Melo et al., 2017).

Besides the effects of LPA on the above mentioned pathways, this lysophospholipid also induces increased expression of matrix metalloproteinase 9 (MMP-9) by ovarian cancer cells (Figure 5; Liu et al., 2012). Overexpression of MMP-9, a zinc-dependent endopeptidase, can have many implications for tumorigenesis and its expression by ovarian cancer cells was associated to increased invasiveness (Hu et al., 2012; Liu et al., 2012). MMP-9 can degrade E-cadherin, a key cell-cell adhesion molecule, being the loss of junctional E-cadherin a crucial step for cancer cells invasion and metastasis, causing loss of epithelial cohesion (Kessenbrock et al., 2010; Hanahan and Weinberg, 2011; Liu et al., 2012). MMP-9 activity can also promote the release of vascular endothelial growth factor (VEGF) from the extracellular matrix, increasing its bioavailability and thus stimulating proangiogenic intracellular pathways (Kessenbrock et al., 2010; Hanahan and Weinberg, 2011). Additionally, the interaction between the CD44 adhesion molecules and the hemopexin-like C-terminal (PEX) domain of MMP-9 on cell membrane can lead to the activation of the tyrosine kinase epidermal growth factor receptor (EGFR), with the consequent phosphorylation of kinase effectors such as FAK, ERK and Akt, thus activating cell signaling cascades that promote cell survival, invasion, migration and platinum resistance (Bauvois, 2012). Interestingly, it was demonstrated that CD44

derived from ovarian cancer cells is internalised by peritoneal mesothelial cells, via uptake of ovarian cancer cells-derived exosomes; exposure to those exosomes induce higher expression of MMP-9 in mesothelial cells, confirming the relevance of CD44-MMP-9 interaction for ovarian cancer tumorigenesis (Nakamura et al., 2017).

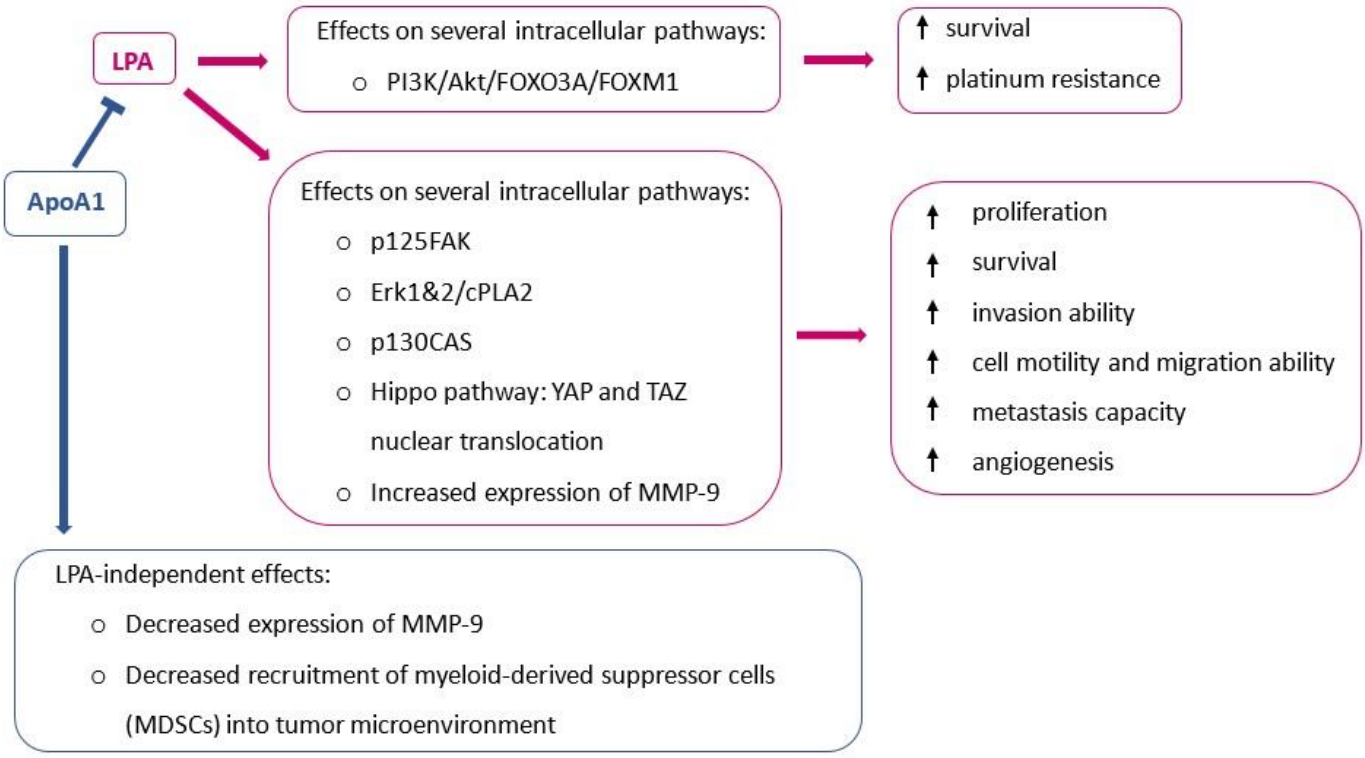

Figure 5 Mechanisms underlying the anti-cancer effects of apolipoprotein A1. *Ovarian cancer cells actively secrete lysophosphatidic acid (LPA), which acts as an autocrine/paracrine factor and stimulates several intracellular pathways that promote ovarian cancer progression and chemoresistance (red boxes). Apolipoprotein A1 (ApoA1) and ApoA1 mimetic peptides have the ability to bind to pro-inflammatory phospholipids, including the oncolipid LPA, consequently avoiding the activation of pro-tumorigenic signalling pathways (Su et al., 2010). ApoA1 and its mimetic peptides can also have anti-cancer effects independently of LPA sequestration (Zamanian-Daryoush et al., 2013; blue box). Abbreviations: cytosolic phospholipase A2 (cPLA2); extracellular signal-regulated kinase 1/2 (ERK1/2); forkhead box protein (FOX); matrix metalloproteinase 9 (MMP-9); p125 focal adhesion kinase (p125FAK); p130 Crk-associated substrate (p130CAS); phosphatidylinositol 3-kinase (PI3K); transcriptional co-activator with PDZ-binding motif (TAZ); Yes-associated protein (YAP).*

Ovarian cancer cells actively secrete LPA, which acts as an autocrine and paracrine factor, stimulating not only the cancer cells but also the stromal cells that comprise the tumor microenvironment (Fang et al., 2002; Jeon et al., 2010). For instance, cancer cells-derived LPA can bind to LPA receptor 1 (LPA1) on mesenchymal stem cells, inducing the secretion of VEGF and thereby further stimulating tumor neoangiogenesis (Sako et al., 2006; Jeon et al., 2010), in addition to the MMP-9-mediated VEGF release from extracellular matrix described above. Because VEGF is known to increase vascular permeability, LPA-induced secretion of this angiogenic factor is thought to be determinant for ovarian cancer progression, namely in terms of ascites formation (Byrne et al., 2003; Li et al., 2009).

Therefore, LPA is implicated in several pathophysiological processes with oncogenic potential that contribute to ovarian cancer progression and resistance to chemotherapy. Importantly, LPA sequestration by ApoA1 or ApoA1 mimetic peptides has been suggested as a potential mechanism for the inhibition of tumor progression *in vivo.* In a transgenic animal model of ovarian cancer, expression of human ApoA1 improves the survival and prevents tumor development, abrogating the LPA-mediated signaling involved in tumor progression and carcinogenesis (Su et al., 2010).

Lysophosphatidic acid-independent anti-tumorigenic effect of apolipoprotein A1

Despite the evidence on LPA relevance for cancer pathogenesis, and the fact that ApoA1 and ApoA1 mimetic peptides can interact with this lysophospholipid, other mechanisms may also contribute to the ApoA1-mediated anti-tumorigenic effect. For instance, Zamanian-Daryoush and collaborators have reported a potent anti-tumorigenic activity of ApoA1, in transgenic animals expressing the human apolipoprotein, after inoculation of melanoma or lung cancer cells; they also demonstrated that subcutaneous administration of ApoA1 was successful in preventing tumor growth and the establishment of metastasis in ApoA1 knockout mice with preexisting melanoma. This effect was completely independent of ApoA1-LPA interaction and there were no changes on LPA plasma levels (Zamanian-Daryoush et al., 2013). Higher ApoA1 expression was associated with lower protein levels and decreased activity of MMP-9,

supporting the hypothesis that ApoA1 may act by other mechanisms than preventing LPA signaling (Figure 5). In addition, ApoA1 also exerted modulatory effects on tumor microenvironment (Figure 5), inhibiting the expansion and recruitment into the tumor bed of myeloid-derived suppressor cells (MDSC) (Zamanian-Daryoush et al., 2013; Zamanian-Daryoush and DiDonato, 2015), a population of cells from myeloid lineage, which expresses both the macrophage marker CD11b and the neutrophil marker Gr-1, and that are associated with immunosuppression, increased angiogenesis and tumor progression (Hanahan and Weinberg, 2011). Therefore, it is plausible that both LPA-dependent (eg. prevention of LPA-mediated activation of signaling pathways) and LPA-independent mechanisms (eg. modulation of tumor microenvironment) may contribute to ApoA1-mediated anti-tumorigenic effect (Figure 5). However, further studies are needed in order to validate the clinical application of ApoA1, ApoA1 mimetic peptides or an ApoA1 booster molecule for treating gynaecologic malignancies or other types of cancer.

2.2. Paraoxonase-1 – antioxidant properties of high density lipoprotein

PON-1 (EC 3.1.8.1) is a Ca^{2+}-dependent HDL-associated aryldialkylphosphatase synthesized primarily by the liver and at lower levels by the colon and kidneys (Deakin et al., 2002; Harel et al., 2004; Costa et al., 2005; Mackness et al., 2010; Soran et al., 2015; Kulka, 2016; Chistiakov et al., 2017; Swedish Human Protein Atlas Project, n.d.). Enzymatically active PON-1 is inserted in the cell membranes of producing tissues, being apolipoproteins-rich HDL particles the physiological acceptors of PON-1, promoting the enzyme detachment from the cell surface (Deakin et al., 2002; Kulka, 2016). PON-1 associates to HDL with very high-affinity and the hydrophobic residues on the N-terminus of PON-1 are involved in its anchoring to the HDL particle (Harel et al., 2004). The presence of ApoA1 in HDL increases PON-1 stability and activity (Gaidukov et al., 2010; Kulka, 2016). Once associated to HDL particles, PON-1 remains in an HDL-bound form in serum or, alternatively, can be redistributed to several tissues without losing its functionality (Deakin et al., 2005; Kulka, 2016; Kunutsor et al., 2016). At least in ovarian

cells, this PON-1 transfer process is reported to be mediated by the SR-B1 receptor (Deakin et al., 2011; Kulka, 2016). The PON-1 redistribution explains the presence of PON-1 protein in several tissues where PON-1 mRNA is not detected (Marsillach et al., 2008; Mackness et al., 2010; Kim et al., 2017; Swedish Human Protein Atlas Project, n.d.).

PON-1 is considered an anti-atherosclerotic enzyme (Chistiakov et al., 2017; Sun et al., 2017) and a free-radical scavenging system (Soran et al., 2015). This enzyme has three different hydrolytic activities identified to date. PON-1 paraoxonase (POase) activity was the first to be studied (Mazur, 1946), and although it is the most commonly employed activity to assess PON-1 status, it mainly reflects PON-1's ability to detoxify environmental toxicants such as organophosphate derivatives and chemical warfare nerve agents (Costa et al., 2005; Valiyaveettil et al., 2012; Bigley and Raushel, 2013; Karlsson et al., 2015). However, the main targets for PON-1-mediated hydrolysis are endogenous toxic compounds. For instance, through its arylesterase (AREase) activity, PON-1 is responsible for detoxifying lipoprotein-associated lipid peroxides and for reducing the lipid peroxide content in the atherosclerotic lesion (Mackness et al., 1993; Aviram et al., 2000; Mehdi and Rizvi, 2012). In fact, higher serum AREase activity was associated with decreased risk for cardiovascular disease and coronary heart disease, after adjustment for several established cardiovascular risk factors (Kunutsor et al., 2016). PON-1 is also involved in the detoxification of homocysteine-thiolactone (Jakubowski, 2000; Perła-Kaján and Jakubowski, 2012), an endogenous reactive specie that can covalently bind to free NH_2 of lysine residues in proteins, generating *N*-homocysteine-protein adducts, in a process designated as protein *N*-homocysteinylation (Perła-Kaján and Jakubowski, 2012; Sharma et al., 2015). The *N*-homocysteinylation process causes structural and functional changes in modified proteins, then inducing endoplasmic reticulum stress and enhancing protein degradation and inflammation (Wu et al., 2015). PON-1, through its lactonase (LACase) activity, is able to hydrolyse homocysteine-thiolactone thus avoiding the accumulation of homocysteine-protein adducts, which have been linked to cardiovascular, autoimmune and neurodegenerative diseases (Jakubowski, 2006; Lakshman et al., 2006; Kamila et al., 2012; Khodadadi et al., 2012; Sharma et al., 2015; Chistiakov et al., 2017). In fact, the LACase activity has been identified as the native activity of PON-1 enzyme

(Khersonsky and Tawfik, 2005). In addition to homocysteine-thiolactone detoxification, it has been suggested that PON-1 LACase activity may play an important role in innate immune system, through enzymatic inactivation of virulence factors (*eg*. *N*-acyl homoserine lactones) (Camps et al., 2011, 2017; Castillo-Juarez et al., 2017), in the hydrolysis of oxidised metabolites of polyunsaturated fatty acids involved in inflammatory responses (Draganov et al., 2005), and also in drug metabolism (*eg*. biotranformation of clopidogrel in its active metabolite) (Bouman et al., 2011; Marchini et al., 2017).

Among the factors that can influence PON-1 levels and activity are polymorphisms of regulatory sequences or in PON-1 coding region (Draganov and La Du, 2004) as well as the exposure to several xenobiotics (Kim et al., 2013). In fact, the interaction between genetic and environmental factors contributes to a final outcome regarding PON-1 status (Deakin et al., 2007). However, it has been recognised that PON-1 genetic variations do not always accurately predict enzyme levels or activity (Jarvik et al., 2000; Kim et al., 2013) and that the contribution of environmental exposures is more significant as a determinant of PON-1 status (Kim et al., 2013). Moreover, pharmacological modulation of PON-1 has been recently suggested as a suitable strategy for improving HDL functionality and antioxidant properties, for therapeutic purposes (Iqbal et al., 2017; Ponce-Ruiz et al., 2017). In fact, low levels of PON-1 enzymatic activities have been observed in several metabolic, degenerative and chronic pathologies (Table 3), particularly in those conditions associated with increased oxidative stress (Camuzcuoglu et al., 2009; Karaman et al., 2010; Balci et al., 2012; Aydin et al., 2013; Bulbuller et al., 2013; Kodydkova et al., 2013; Kirbas et al., 2014; Malik et al., 2014; Sehitogulları et al., 2014; Korkmaz et al., 2015). Furthermore, and regarding oncologic diseases, high levels of serum malondialdehyde (MDA) were reported in patients suffering from several types of malignancies (Karaman et al., 2010; Malik et al., 2014; Sehitogulları et al., 2014). MDA is a secondary product of lipid peroxidation (Slatter et al., 1999; Ayala et al., 2014), which can form adducts with several biomacromolecules, namely with DNA (Ayala et al., 2014). This aldehyde is not a substrate of PON-1, but it accumulates in consequence of high lipid peroxidation rates that are not compensated by PON-1 detoxification (Ayala et al., 2014). MDA has been identified as highly mutagenic for human cells (Niedernhofer et al., 2003), and

implicated in cancer pathogenesis (Ayala et al., 2014; Martinez-Useros and Garcia-Foncillas, 2016). The imbalance in oxidation-reduction status, towards the preferential formation of free radicals that is not compensated by the antioxidant defence, is widely recognised as a causal factor involved in the onset of carcinogenesis (Pisoschi and Pop, 2015). Besides promoting oxidative damage to macromolecules, reactive oxygen species can also activate tumorigenic signalling, particularly through the PI3K/Akt and hypoxia-inducible factor (HIF)-1α intracellular pathways (Sullivan and Chandel, 2014). Since PON-1 is a multifunctional antioxidant enzyme comprising a scavenger system, a decrease of PON-1 activities in pathologic conditions in which oxidative stress plays a central role would be expected (Table 3). Therefore, the therapeutic modulation of PON-1 might benefit patients with different diseases.

Table 3. Paraoxonase-1 status in pathologic conditions. *The paraoxonase-1 (PON-1) status in some metabolic, degenerative and chronic diseases is presented below as the measurement of serum PON-1 paraoxonase (POase), arylesterase (AREase) and/or lactonase (LACase) activities in affected populations.*

Pathology		***PON-1 status***	***Reference***
Cardiovascular and metabolic disorders	***Acute myocardial infarction***	Lower POase activity in patients with acute myocardial infarction, comparatively with patients with coronary artery disease with no major coronary events.	Maturu et al., 2013
	Diabetic retinopathy	Lower POase activity in patients with diabetic retinopathy, comparatively with patients with non-complicated diabetes.	Mackness et al., 2000
	Obesity/diabetes	Lower AREase and LACase activities among populations with higher prevalence of diabetes and obesity.	Gugliucci et al., 2015
Neurodegenerative conditions	***Parkinson's disease***	Lower POase and AREase activities in patients with Parkinson's disease.	Kirbas et al., 2014
	Alzheimer's disease	Lower POase, AREase and LACase activities in patients with Alzheimer's disease.	Bacchetti et al., 2015

Malignant diseases	***Oral squamous cell carcinoma***	Lower POase and AREase activities in cancer patients. .	Malik et al., 2014
	Esophageal squamous cell carcinoma		Sehitogulları et al., 2014
	Laryngeal squamous cell carcinoma		Karaman et al., 2010
	Papillary thyroid cancer		Korkmaz et al., 2015
	Lung cancer		Balci et al., 2012
	Breast cancer		Balci et al., 2012
	Ovarian cancer		Camuzcuoglu et al., 2009
	Colorectal cancer		Balci et al., 2012; Bulbuller et al., 2013
	Pancreatic cancer	Lower AREase and LACase activities in cancer patients.	Kodydkova et al., 2013
	Bladder cancer	Lower AREase activity in cancer patients.	Aydin et al., 2013
	Acute myeloid leukemia		Çebi et al., 2015

3. Nevirapine pharmacokinetics and toxicology

3.1. A focus on nevirapine pharmacokinetics

NVP (Figure 6) is a highly lipophilic drug, being extensively absorbed after oral administration, with a bioavailability of 90% (Smith et al., 2001; Macha et al., 2009). After continuous administration of a daily dose of 400 mg, the steady-state maximum plasma concentration (Cmax) and trough concentration (Cmin) are approximately 7.2 mg/L and 4.0 mg/L, respectively (Fumero and Podzamczer, 2001; Marinho et al., 2014c). Secondary peaks after Cmax were observed in some NVP pharmacokinetic studies, which indicate that NVP may possibly enter enterohepatic cycling (Havlir et al., 1995; Fumero and Podzamczer, 2001) or undergo gastric secretion with posterior intestinal reabsorption as suggested by Ibarra and collaborators (2014). NVP binding to albumin is approximately 60% (Smith et al., 2001; Bocedi et al., 2004), this drug easily crosses the placenta and the blood-brain barrier, being one of the antiretrovirals that reaches highest concentrations in the cerebrospinal fluid (Smith et al., 2001; Calcagno et al., 2014).

In phase I metabolic reactions, NVP is extensively biotransformed by different cytochrome P450 (CYP450) isoforms, leading to the formation of several phase I hydroxylated metabolites: 2-hydroxy-nevirapine (2-OH-NVP), 3-hydroxy-nevirapine (3-OH-NVP), 8-hydroxy-nevirapine (8-OH-NVP) and 12-hydroxy-nevirapine (12-hydroxy-NVP). The 12-OH-NVP metabolite can be further oxidised to generate 4-carboxy-nevirapine (4-COOH-NVP), another NVP phase I metabolite (Erickson et al., 1999; Riska et al., 1999; Chen et al., 2008; Grilo et al., 2013; Marinho et al., 2014b; Pinheiro et al., 2017). Different CYP450 pathways are implicated in the formation of each NVP phase I metabolite (Figure 7). For instance, 2-OH-NVP is formed by CYP3A4 and CYP3A5-mediated metabolism, while CYP2B6 is the only isoform involved in the formation of 3-OH-NVP. The formation of 8-OH-NVP was attributed mainly to CYP2D6, with contributions of CYP2B6 and CYP3A4 to a lesser extent. The NVP main phase I metabolite, 12-OH-NVP, is primarily formed through CYP3A4 metabolism, although CYP3A5, CYP2D6 and CYP2C9 can also contribute for its formation (Erickson et al., 1999; Grilo et al., 2013). NVP is an inducer of CYP3A4 and CYP2B6, through activation of constitutive androstane receptor (CAR), and can thereby induce its own

biotransformation (Faucette et al., 2006). This NVP auto-induction effect is complete within 28 days of daily administration, when the drug reaches the steady-state plasma concentrations (Lamson et al., 1999; Faucette et al., 2006). Moreover, in addition to hepatic metabolism, our research group have demonstrated the intestinal contribution to NVP phase I biotransformation as a relevant source of inter-individual variability on NVP pharmacokinetics (Pinheiro et al., 2015).

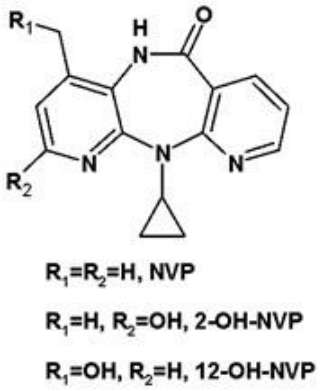

Figure 6. Structures of nevirapine and its two main phase I metabolites, 2-hydroxy-nevirapine and 12-hydroxy-nevirapine. *NVP, nevirapine; 2-OH-NVP, 2-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine. In: Marinho et al., 2016.*

NVP phase I metabolites can be further metabolised in subsequent phase II reactions, such as glucuronidation, catalysed by uridine-diphosphate-glucuronosyltransferases (UGTs). In fact, glucuronide conjugates of 2-, 3-, 8- and 12-OH-NVP have been identified in three-dimensional (3D) cultures of hepatocytes (Pinheiro et al., 2017), in urine and bile of animals exposed to NVP (Srivastava et al., 2010) and in urine of NVP-treated patients (Riska et al., 1999b; Srivastava et al., 2010). The renal excretion of glucuronides is a major route for NVP elimination (Riska et al., 1999b), although the UGT isoforms involved in NVP biotransformation have not yet been identified. In addition to glucuronidation, other pathways might contribute for NVP phase II biotransformation, namely sulfoconjugation (Pinheiro et al., 2017) and conjugation with glutathione (Srivastava et al., 2010; Dekker et al., 2016). It has been increasingly recognised that the relative contribution of each phase I and II biotransformation pathways can have a determinant impact on the kinetics of NVP reactive metabolites, and consequently on the development of NVP-induced toxic reactions (Figure 7).

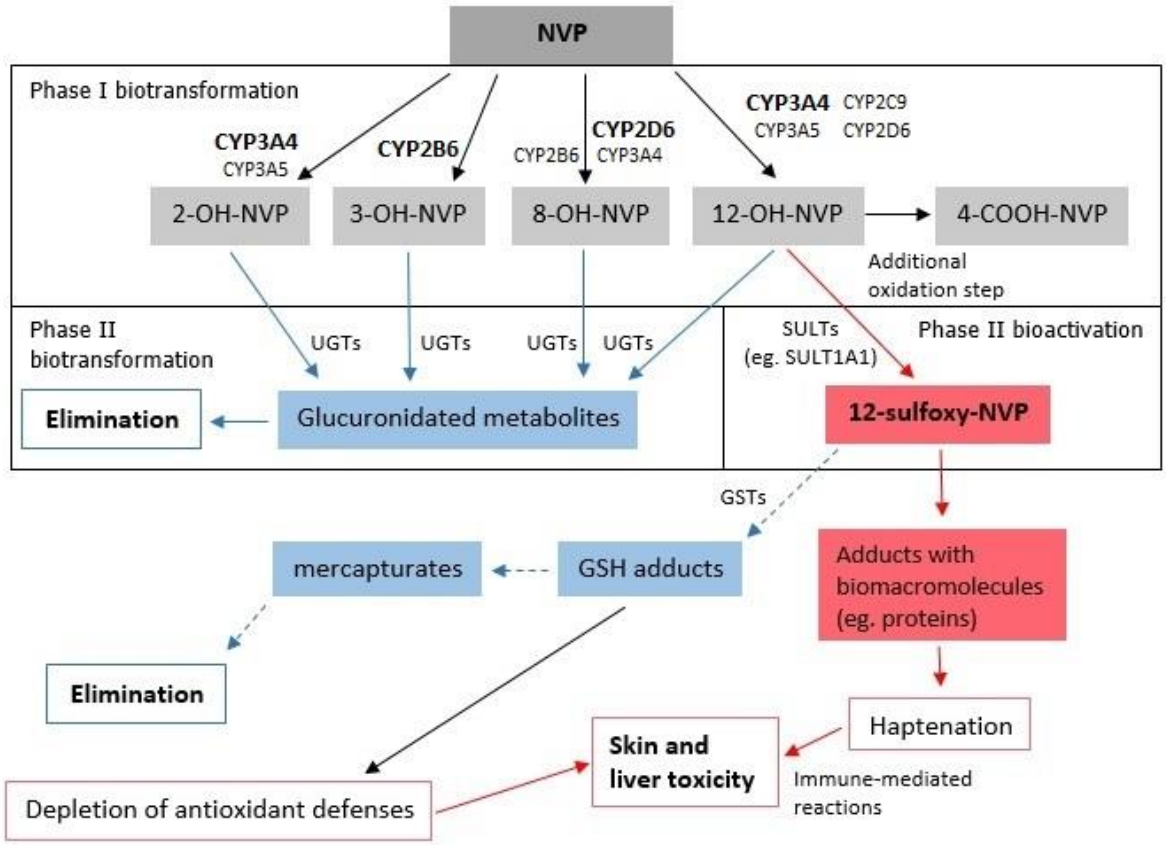

Figure 7 Nevirapine biotransformation and bioactivation pathways. *Several isoforms of cytochrome P450 (CYP450) contribute to nevirapine (NVP) phase I biotransformation, generating NVP hydroxylated metabolites (Erickson et al., 1999): 2-, 3-, 8- and 12-hydroxy-nevirapine (-OH-NVP). The 12-OH-NVP metabolite undergoes an additional oxidation step yielding 4-carboxy-nevirapine (4-COOH-NVP) (Chen et al., 2008). Glucuronidation of phase I metabolites represents a major pathway for NVP elimination (solid blue arrows; Riska et al., 1999). Other phase II reactions may also occur, namely sulfotransferases (SULTs)-mediated bioactivation of 12-OH-NVP, with formation of 12-sulfoxy-NVP (Sharma et al., 2013), a reactive electrophile which is prone to form adducts with macromolecules (solid red arrows; Caixas et al., 2012), namely with proteins and DNA (Antunes et al., 2008, 2010b). Other bioactivation pathways have been proposed, however 12-OH-NVP sulfonation is the most studied pathway and its relevance for NVP-induced toxicity has been confirmed in animal models and in clinical studies. Alternatively, reactive metabolites can form adducts with glutathione (GSH) being eliminated in the urine as mercapturates (dashed blue arrows; Srivastava et al., 2010). However, it seems implausible that GSH conjugation contributes for NVP detoxification at the same extent than glucuronidation, due to the kinetic parameters of the reaction catalysed by glutathione S-transferases (GSTs) (Dekker et al., 2016). Adapted from Marinho et al., 2014b.*

3.2. Nevirapine toxicology: the importance of the balance between bioactivation and detoxification pathways

NVP treatment has been associated with severe idiosyncratic hepatotoxicity and skin rash. Several risk factors for NVP-induced toxic reactions have been reported, such as female sex (Antinori et al., 2001; Bersoff-Matcha et al., 2001; Kesselring et al., 2009; Günthard et al., 2014; Pawar, 2015), higher $CD4^+$ cell counts (Kiertiburanakul et al., 2008; Kesselring et al., 2009; Günthard et al., 2014), detectable HIV-1 viral load (Kesselring et al., 2009), Asian ethnicity (Ho et al., 1998; Kesselring et al., 2009), pregnancy (Bera and Mia, 2012; Huntington et al., 2014), history of drug allergies (Kiertiburanakul et al., 2008) and also concomitant treatment with corticosteroids or trimethoprim/sulfamethoxazole (Antinori et al., 2001; Tseng et al., 2014). In an attempt to minimize the risk of NVP-induced toxicity, it was recommended a cut-off relatively to $CD4^+$ cell counts, which varies according to sex. Thus, NVP must be initiated in HIV-infected women with $CD4^+$ counts below 250 cells/mm^3 and in HIV-infected men with $CD4^+$ counts below 400 cells/mm^3 (Günthard et al., 2014; World Health Organization, 2016).

In recent years, the molecular pathways leading to NVP toxic reactions have been extensively studied and are now better understood (Figure 7). Solid evidence has emerged on the role of NVP bioactivation into reactive metabolites as a causal factor for the onset of NVP-related toxicity (Antunes et al., 2008, 2010a, 2010b, 2011; Chen et al., 2008; Wen et al., 2009; Srivastava et al., 2010; Caixas et al., 2012; Meng et al., 2013; Sharma et al., 2013; Kranendonk et al., 2014; Marinho et al., 2014a, 2014b; Pinheiro et al., 2015, 2017; Dekker et al., 2016). Although unstable electrophiles can be generated by different metabolic pathways (Wen et al., 2009; Srivastava et al., 2010; Antunes et al., 2011; Dekker et al., 2016), the SULTs-mediated phase II biotransformation of 12-OH-NVP seems to be the most relevant mechanism for NVP toxicity (Antunes et al., 2008, 2010a, 2010b, 2011; Chen et al., 2008; Wen et al., 2009; Srivastava et al., 2010; Caixas et al., 2012; Meng et al., 2013; Sharma et al., 2013; Kranendonk et al., 2014; Marinho et al., 2014a, 2014b; Pinheiro et al., 2015, 2017; Dekker et al., 2016). In fact, SULTs-mediated metabolism of 12-OH-NVP leads to the formation of 12-sulfoxy-NVP, which can bind covalently to biomacromolecules yielding adducts with DNA (Antunes et al., 2008), aminoacids (Antunes et al., 2010a) and proteins (Antunes et al., 2010b; Caixas et

al., 2012; Meng et al., 2013; Sharma et al., 2013; Pinheiro et al., 2017). The identification of these 12-sulfoxy-NVP-derived adducts in NVP-treated patients further supports the relevance of this bioactivation pathway (Caixas et al., 2012; Meng et al., 2013). SULTs-mediated sulfonation is an essential process in phase II biotransformation of several xenobiotics (Gamage, 2005). These reactions occur in the presence of the SULTs' cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the donor of the sulfonate (SO_3^-) group (Gamage, 2005; Suiko et al., 2017). The biosynthesis of PAPS comprises two sequential steps, the first one is performed by an ATP-sulfurylase, generating adenosine 5'-phosphosulfate (APS), and the following step involves APS phosphorylation by an APS kinase, resulting in the formation of PAPS (Venkatachalam et al., 1998; Fuda et al., 2002; Coughtrie, 2016). PAPS biosynthesis in man is catalysed by PAPS synthase 1 and 2; both of the enzymes have ATP-sulfurylase and APS kinase activities (Venkatachalam et al., 1998; Fuda et al., 2002; Coughtrie, 2016). PAPS synthase 2, which presents significantly higher catalytic efficiency compared with PAPS synthase 1 (Fuda et al., 2002), has a female predominant expression in liver (Alnouti and Klaassen, 2006, 2011). It is also noteworthy that all hepatic SULTs with differential expression between sexes (*eg*. SULT1A1, SULT1C2, SULT1D1, SULT2A1, SULT2A2, SULT3A1) are female predominant, with the only exception of SULT1C1 which has higher expression in male liver (Tsoi et al., 2001; Wu et al., 2001; Alnouti and Klaassen, 2006, 2011; Hirao et al., 2011; Suzuki et al., 2012; Chen et al., 2017). These sex differences in the expression of hepatic SULTs have been related to suppressive effects of androgens and stimulatory effects of oestrogens as well as differential modulation of hepatic SULTs' expression induced by male- and female-patterns of growth hormone secretion (Alnouti and Klaassen, 2011). Importantly, one of the female predominant SULTs, SULT1A1, can definitely promote sulfoconjugation of 12-OH-NVP (Sharma et al., 2013; Kranendonk et al., 2014). This specific SULT is expressed at high levels in human liver and skin, the target tissues for NVP toxicity (Dooley et al., 2000; Hempel et al., 2007). Moreover, 12-OH-NVP is the metabolite in higher proportion in plasma from NVP-treated women (Marinho et al., 2014c), which means higher availability of substrate for SULT1A1 in females. Besides, another major NVP metabolite, 2-OH-NVP, is able to induce hepatic SULT1A1 activity as demonstrated in a 3D model of hepatocytes (Pinheiro et al., 2017); therefore 2-OH-NVP formation can further contribute to 12-OH-NVP bioactivation. Also, the male

predominant expression of some UGTs that can promote glucuronidation and subsequent biliary or urinary elimination of phenolic substrates (Gallagher et al., 2010; Wu et al., 2011; Chen et al., 2017), might contribute to sex differences in NVP detoxification, by preferential glucuronide conjugation of phase I (phenolic) NVP metabolites in men. Taken together, this evidence points towards an imbalance between the formation of 12-sulfoxy-NVP and detoxification pathways in female sex. This imbalance will favour the accumulation of NVP-derived reactive species and the emergence of toxic reactions among women, providing an explanation for the sex-dependent dimorphic profile of NVP-induced toxicity. Besides sex, another risk factors for NVP-induced toxicity can be mechanistically explained by this toxicokinetic hypothesis. For instance, pregnancy is associated with a decreased ability for hepatic glucuronidation of phenolic compounds, and increased sulfoconjugation ability caused by higher hepatic mRNA and protein levels of SULT1A1 (Wen et al., 2013; Bright et al., 2016). Likewise, co-treatment with corticosteroids (*eg*. dexamethasone, prednisolone) might increase the risk of NVP toxicity by an induction effect on hepatic expression of SULT1A3 and SULT2A1 (Duanmu et al., 2002; Bian et al., 2007). Although it is not known if these specific SULTs can contribute to the formation of 12-sulfoxy-NVP, this assumption seems at least plausible due to the significant substrate overlapping between different SULTs (Barnett et al., 2004; Gamage, 2005). The higher incidence of NVP-induced toxic reactions among Chinese patients (Ho et al., 1998) can also be explained by this SULT1A1/12-sulfoxy-NVP putative model. In fact, SULT1A1 is a highly polymorphic sulfotransferase, and the SULT1A1*1 allele is associated with higher protein stability and higher catalytic activity toward several substrates (Nagar et al., 2006). The SULT1A1*1 frequency in the Chinese population was reported to be significantly higher than the allele frequency in African Americans or Caucasians (Carlini et al., 2001). Therefore, it is reasonable to assume that different genetic backgrounds, particularly polymorphisms affecting enzymes involved in NVP pharmacokinetics and bioactivation might influence the risk of NVP-related toxicity.

As discussed above, NVP biotransformation into 12-OH-NVP followed by subsequent SULT-mediated bioactivation into 12-sulfoxy-NVP (Figure 7) seems to be the most relevant molecular pathway leading to the onset of NVP toxic reactions. However, other

NVP-derived reactive metabolites have been proposed. For instance, a reactive quinone methide formed through the loss of sulphate group of 12-sulfoxy-NVP (Chen et al., 2008), or alternatively in the absence of phase II biotransformation, through CYP450-mediated NVP dehydrogenation (Wen et al., 2009), is thought to be the metabolite trapped by the antioxidant tripeptide glutathione (GSH), generating NVP-12-GSH conjugates (Wen et al., 2009; Srivastava et al., 2010). Although the 12-sulfoxy-NVP metabolite was also shown to generate NVP-12-GSH in a reaction catalysed by glutathione *S*-transferases (GSTs) GSTM1-1, GSTA1-1 or GSTA3-3 (Dekker et al., 2016). Another metabolite involved in the formation of GSH adducts is NVP-2,3-epoxide, an intermediate in 2-OH-NVP formation. This epoxide can generate a NVP-3-GSH conjugate (Srivastava et al., 2010; Dekker et al., 2016) in a reaction catalysed by GSTP1-1 (Dekker et al., 2016). GSH conjugation is an important phase II detoxification pathway for several endo- and xenobiotics, that allows urinary elimination of the respective mercapturates, preventing damage of macromolecules induced by reactive species (Eaton and Bammler, 1999; Grillo, 2015; Mathias and B'hymer, 2016). Two NVP-derived mercapturates, NVP-3-mercapturate and NVP-12-mercapturate, were identified in the urine of animals and HIV-infected patients (Srivastava et al., 2010), further confirming the occurrence of GSH conjugation of NVP metabolites (Figure 7). However, as recently demonstrated, while these mercapturates have a significant value as biomarkers for assessing exposure to electrophiles, this pathway is unlike to significantly contribute to the detoxification of NVP reactive derivatives, due to the low activity of GSTs catalysis and slow kinetic reaction (Dekker et al., 2016). Moreover, GSH-mediated detoxification pathways are thought to be impaired in the context of HIV infection, as decreased synthesis and lower levels of GSH were reported in these patients (Smith et al., 1996).

In addition to the previously mentioned reactive NVP metabolites (12-sulfoxy-NVP, NVP quinone methide and NVP-2,3-epoxide), it was demonstrated that chemical and enzymatic oxidation of 2-OH-NVP can generate a reactive quinone-imine *in vitro* (Antunes et al., 2011). However, the relevance of this quinone-imine for NVP-induced toxicity has yet to be confirmed *in vivo*, particularly because most of the 2-OH-NVP metabolite undergoes preferentially phase II UGTs and SULTs metabolic pathways (Pinheiro et al., 2017).

3.3. Nevirapine immunotoxicology

NVP-induced skin rash and hepatotoxicity are immune-mediated idiosyncratic reactions (Shenton et al., 2005; Popovic et al., 2006, 2010; Kiertiburanakul et al., 2008; Kesselring et al., 2009; Ng et al., 2012; Günthard et al., 2014; World Health Organization, 2016), which can range from relatively mild forms to severe clinical manifestations, such as toxic epidermal necrolysis, Stevens-Johnson syndrome or fulminant liver failure (Lopez-Delgado et al., 2012; Paik, 2016).

Regarding the immune-mediated mechanism of NVP toxicity, two different pathways have been suggested for hepatotoxicity and skin rash (Yuan et al., 2011; Keane et al., 2014). Hepatotoxic reactions seem to involve antigen presentation to $CD4^+$ T lymphocytes through major histocompatibility complex (MHC) class II, while cutaneous adverse events are probably due to antigen presentation to $CD8^+$ T cells through MHC class I. In fact, several studies have associated MHC class I (or human leukocyte antigen (HLA)-A, -B and -C) and MHC class II (or HLA-DR) alleles to different phenotypes relatively to NVP toxicity. For instance, HLA-B*3505 and HLA-Cw*0401 were linked with increased risk of NVP induced rash, including Stevens-Johnson syndrome and toxic epidermal necrolysis (Chantarangsu et al., 2009; Likanonsakul et al., 2009; Arab-Alameddine et al., 2011; Yuan et al., 2011; Carr et al., 2013; Keane et al., 2014) while HLA-DRB1*0101 was associated with increased risk of hepatotoxicity (Martin et al., 2005; Arab-Alameddine et al., 2011; Yuan et al., 2011; Keane et al., 2014). Haptenation of self-proteins by NVP reactive metabolites, namely 12-sulfoxy-NVP (Antunes et al., 2010a, 2010b; Caixas et al., 2012; Meng et al., 2013; Sharma et al., 2013; Pinheiro et al., 2017) seems to be the link between NVP bioactivation and the immune-mediated mechanism underlying NVP-induced toxicity, since the covalent modification of proteins can change their normal function and trigger an immune response against the modified protein (House et al., 2014). Moreover, the persistent inflammatory status and chronic immune activation observed in the context of HIV infection might contribute to immune-mediated toxicity (Aberg, 2012).

GENERAL OBJECTIVES

The general goal of the current work was to provide new and useful insights that can contribute to the future development of an HDL booster drug. A drug able to modify and improve HDL functionality might be a valuable therapeutic resource, for treating diseases for which the therapeutic arsenal is still limited.

NVP treatment has been associated with significant increases in HDL-cholesterol and ApoA1 levels (Table 1). This positive effect on HDL is probably intrinsic to NVP and not related with control of viral replication, as observed in uninfected newborns receiving NVP for HIV prophylaxis (Sankatsing et al., 2007) and in HIV-infected patients with stably suppressed viral load (Franssen et al., 2009). Moreover, HDL increases induced by NVP treatment translate into clinical benefits on atherosclerosis progression (Maggi et al., 2011; Gleason et al., 2016), unlike the changes in lipid profile induced by HDL-targeted therapies, such as niacin (The AIM-HIGH Investigators, 2011; The HPS2-THRIVE Collaborative Group, 2014), the new generation CETP inhibitor evacetrapib (Eyvazian and Frishman, 2017) or the BET protein inhibitor RVX-208 (Nicholls et al., 2016), which were associated with no clinical benefits. In fact, despite significant investments on the development of HDL booster molecules, there are currently no drugs for effectively increasing HDL levels and improving HDL function (Table 1).

The main disadvantage of NVP is its association with potentially life-threatening skin and liver toxicity, which can hamper its re-profiling into an HDL booster molecule. However, compelling evidence has shown that NVP toxic reactions are strictly dependent on drug biotransformation and bioactivation through specific metabolic pathways (Antunes et al., 2008, 2010a, 2010b; Caixas et al., 2012; Marinho et al., 2014c; Pinheiro et al., 2017).

Our hypothesis is that one of NVP metabolites, with a better toxicokinetic profile, might be the responsible for the NVP-induced HDL booster effect. Thus, the central research question that drives the next three chapters of the present dissertation is the following: *To what extent does biotransformation contribute to the HDL modulation induced by NVP?* Given these premises, the primary specific objective of the work reported in here was to reveal the relations between NVP biotransformation and HDL modulation, by focusing on the effect of NVP and its metabolites on ApoA1 and PON-1, two components of HDL proteome critically involved on HDL particle functionality. Additionally the assessment of auto-antibodies against HDL particle and ApoA1 can provide valuable

insights on the modulation of HDL dysfunctionality (Batuca et al., 2007, 2016). To achieve this goal, a translational approach strategy was conducted, resorting to exploratory prospective and cross-sectional clinical studies (*cf.* Chapter 1), complemented by *in vitro* studies (*cf.* Chapters 2 and 3) that allowed the clarification of the individual contribution of each NVP metabolite/NVP biotransformation pathway in the modulation of ApoA1 and PON-1.

Finally, considering NVP's female-predominant toxic profile (Antinori et al., 2001; Bersoff-Matcha et al., 2001; Marinho et al., 2014c), its boosting capability on ApoA1 (Franssen et al., 2009) and its anti-tumorigenic efficacy (Mangiacasale et al., 2003; Hecht et al., 2015), we hypothesised that this drug might be useful for ovarian cancer. For this reason, and considering that the emergence of chemoresistance is one of the major challenges in the management of ovarian cancer, our secondary specific objective was to investigate the relevance of ApoA1 in ovarian cancer treatment, particularly concerning its potential for chemosensitisation. For studying the anti-tumorigenic properties of ApoA1, we employed *in vitro* cultures of ovarian cancer cell lines and also the more biologically relevant *in ovo* xenograft model (*cf.* Chapter 4).

CHAPTER 1. MODULATION OF HIGH DENSITY LIPOPROTEIN FUNCTIONALITY BY NEVIRAPINE: REVEALING THE SIGNIFICANCE OF DRUG BIOTRANSFORMATION

Chapter 1

Modulation of high density lipoprotein functionality by nevirapine: revealing the significance of drug biotransformation

1.1. Summary

NVP is a widely used antiretroviral associated with biotransformation-driven female-predominant toxicity. Despite this, NVP's efficacy and lipid-friendly properties granted this drug a prominent role in HIV treatment. The aim of the current study was to investigate the relations between NVP treatment, NVP biotransformation and HDL functionality.

In order to achieve this aim, a prospective and a cross-sectional study were performed. The end-points evaluated in these studies were HDL-cholesterol, ApoA1, anti-HDL and anti-ApoA1 antibodies, POase, AREase and LACase activities of PON-1 enzyme. Plasma levels of NVP and its metabolites were quantified by HPLC. Serum PON-1 POase, AREase and LACase activities were quantified spectrophotometrically. ApoA1 plasma levels were quantified by an immunoturbidimetric assay. Serum levels of anti-HDL and anti-ApoA1 antibodies were assessed by ELISA.

Eleven HIV-infected patients starting NVP-based cART were prospectively followed for up to 20 weeks. A total of 146 cART-naïve patients and 186 NVP-treated patients (400 mg NVP/day for at least 3 months) were included in the cross-sectional study. NVP treatment was associated with higher levels of HDL-cholesterol and ApoA1, higher PON-1 POase, AREase and LACase activities, and lower levels of anti-HDL and anti-ApoA1 antibodies. In the prospective study was evident a temporal modulation of HDL functionality. The earliest event observed was the decrease in anti-HDL antibodies, while modulation of the other end-points was later observed. In the cross-sectional analysis, women on NVP treatment was the group with higher levels of HDL-cholesterol and ApoA1 ($p< 0.05$). In a multivariable analysis, the proportions of 3-OH-NVP ($p= 0.009$), 12-OH-NVP ($p= 0.007$) as well as the proportion of 2-OH-NVP adjusted by body weight ($p= 0.005$) were negatively associated with PON-1 LACase activity. The proportion of 2-OH-NVP was also strongly associated with lower levels of anti-HDL antibodies ($p= 0.03$). Regarding the relations between NVP and its metabolites, NVP plasma levels were

associated with 2-OH-NVP (p= 0.03), 12-OH-NVP (p< 0.001) and particularly with 3-OH-NVP (p< 0.001); while the proportion of 3-OH-NVP was negatively associated with the proportions of 2-OH-NVP (p< 0.001) and 12-OH-NVP (p< 0.001).

This metabolite profile may be consistent with NVP-induced CAR activation and the late modulatory effects on HDL functionality suggest the accumulation of a NVP metabolite with more lipid-friendly properties than NVP. The evidence herein gathered supports that NVP treatment beneficially modulates HDL functionality, being NVP biotransformation a possible factor affecting these modulatory effects.

1.2. Objectives:

The aim of the current study was to disclose the relations between NVP treatment, NVP biotransformation, lipid profile and HDL functionality in HIV-infected patients. In order to achieve this aim, we performed a prospective analysis with patients starting NVP-based antiretroviral treatment and a cross-sectional study including cART-naïve patients and patients on long-term NVP treatment.

1.3. Methods

1.3.1. Study design

A prospective and a cross-sectional clinical investigation were conducted. Both clinical studies were performed in accordance with the principles stated in the Declaration of Helsinki. The experimental protocols were approved by the National Committee for Data Protection (*Comissão Nacional de Protecção de dados*; process number 6567/2009) and by the Ethics Committees of the hospitals involved (*Centro Hospitalar de Lisboa Central, EPE*, process number 32-CHLC and *Hospital Professor Doutor Fernando Fonseca, EPE,* process number CA21/2011). Prior to inclusion in the studies, all patients voluntarily gave their written informed consent. All included patients were adults with diagnosed HIV-1 infection. Exclusion criteria applied in this study were being under 18 years of age, having AIDS-defining conditions, having compliance issues and being on antidyslipidemic therapy.

Prospective study

HIV-1 infected patients starting NVP-based cART, either or not as first cART scheme, were included in the prospective study and followed during four visits: first visit, before

starting NVP-based cART (baseline); second visit (2 weeks of treatment), third visit (4 weeks of treatment) and fourth visit (between 8 and 20 weeks of treatment). NVP schedule was performed according to international guidelines: in the first two weeks of treatment, NVP was administered at a daily dose of 200 mg, followed by a maintenance dose of 400 mg/day. NVP is initiated in HIV-infected women with $CD4^+$ counts below 250 cells/mm^3 and in HIV-infected men with $CD4^+$ counts below 400 cells/mm^3 (Günthard et al., 2014; World Health Organization, 2016). Adherence to antiretroviral therapy was controlled by the clinicians. The study end-points were HDL-cholesterol, ApoA1, anti-HDL antibodies, anti-ApoA1 antibodies, PON-1 POase, AREase and LACase activities. A schematic representation of the prospective study design is presented in Figure 8. The following data were collected for each patient: sex, age, ethnicity, body weight, height, prior cART experience, time between blood sampling and last NVP intake, cART backbone, HIV-1 viral load, $CD4^+$ cell counts, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALKP). Blood samples (2mL) for ApoA1 quantification were collected into EDTA-containing tubes, while blood samples (2mL) for quantifications of PON-1 activities, anti-HDL and anti-ApoA1 antibodies were collected into tubes with no anti-coagulant.

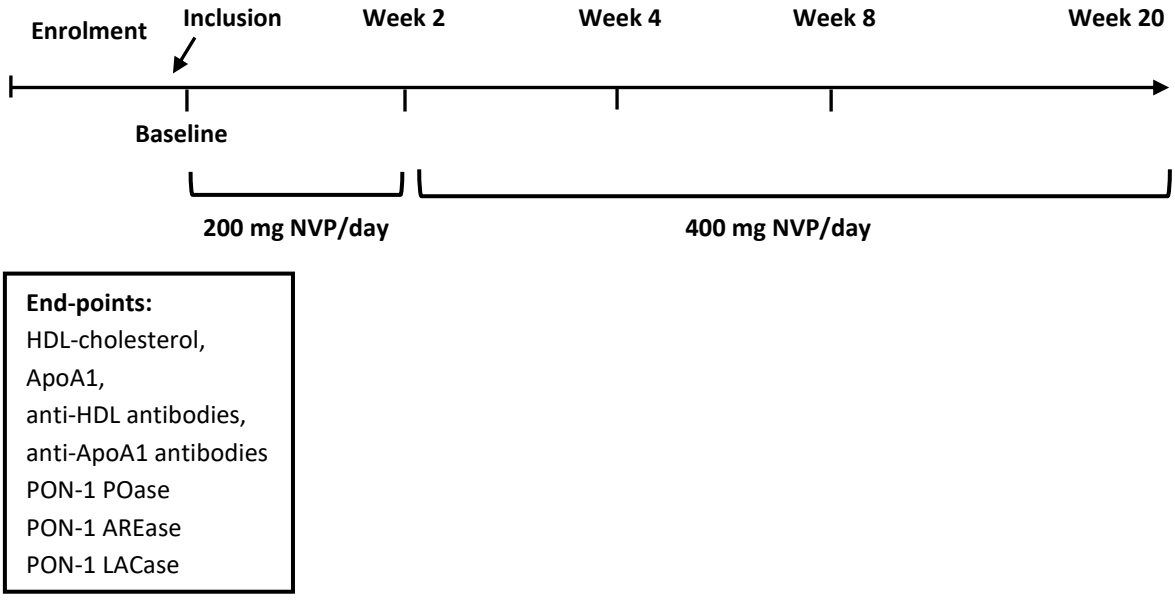

Figure 8 Schematic representation of the prospective study design. *After the eligibility assessment, patients starting nevirapine (NVP)-based combined antiretroviral therapy (cART) were included in the prospective study. NVP is initiated in HIV-infected women with $CD4^+$ counts below 250 cells/mm^3 and in HIV-infected men with $CD4^+$ counts below 400 cells/mm^3. Patients were followed during four visits: Before starting NVP-based cART (baseline), two weeks after starting NVP treatment, at a daily dose of 200 mg (week 2), four weeks after starting NVP treatment – consisting of a period of two weeks on 200 mg NVP/day followed by two weeks on 400 mg NVP/day (week 4) and the last visit, between 8 and 20 weeks after starting NVP-based cART. The end-points evaluated in this prospective analysis were high density lipoprotein (HDL)-cholesterol, apolipoprotein A1 (ApoA1), anti-HDL antibodies, anti-ApoA1 antibodies, paraoxonase-1 (PON-1) paraoxonase (POase), arylesterase (AREase) and lactonase (LACase) activities.*

Cross-sectional study

Two groups of patients were included in the cross-sectional study. The naïve group was comprised by HIV-1 infected patients without previous antiretroviral experience. The NVP group included patients who had been treated with a NVP-based cART scheme for more than three months, irrespectively of previous cART experience. Adherence to antiretroviral therapy was controlled by the clinicians.

The following data were collected for each patient: sex, age, ethnicity, body weight, height, time on cART, time on NVP-based cART, time between blood sampling and last NVP intake, cART backbone, HIV-1 viral load, $CD4^+$ cell counts, ALT, LDH, GGT, ALKP, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. The study end-points were HDL-cholesterol, ApoA1, anti-HDL antibodies, anti-ApoA1 antibodies, POase, AREase and LACase activities of PON-1.

Blood samples (2mL) for NVP and NVP metabolites quantification and for ApoA1 quantification were collected into EDTA-containing tubes, while blood samples (2mL) for quantifications of PON-1 activities, anti-HDL and anti-ApoA1 antibodies were collected into tubes with no anti-coagulant.

Additionally, HIV-infected patients on EFV- and PIs-based cART schemes were also included in a cross-sectional analysis of PON-1 POase activity and anti-HDL antibodies, in order to elucidate whether the NVP-induced effect on HDL is a drug-specific effect or a consequence of the suppression of HIV replication.

1.3.1. Quantification of nevirapine and its metabolites

Plasma was obtained by centrifugation of blood samples from EDTA-containing tubes at 3000 *g* for 10 min. A volume of 900 µL of plasma was heated at 60 ˚C for 1 hour for viral inactivation. NVP and its phase I metabolites were then extracted from plasma using dichloromethane partitioning. The analytes were quantified by a fully validated in-house developed high performance liquid chromatography (HPLC) method (Marinho et al., 2014d). The plasma levels of NVP and its metabolites were expressed in ng/mL.

1.3.2. Quantification of paraoxonase-1 activities

Serum was obtained by centrifugation of blood samples from serum tubes, with no anticoagulant, at 3000 *g* for 10 min. The quantification of PON-1 activities was performed by in-house developed and validated methods, as previously described by Dias and collaborators (2014a, 2014b). Briefly, for the quantification of PON-1 POase activity, the substrate employed was paraoxon and the formation of *p*-nitrophenol was spectrophotometrically monitored; for PON-1 AREase activity the substrate used was phenyl acetate, being monitored the hydrolysis of this substrate into acetic acid; while for the LACase activity of PON-1 the substrate was dihydrocoumarin and the product monitored was 3-(o-hydroxyphenyl) propionic acid (Figure 9). PON-1 POase activity was expressed in $U.L^{-1}$, while AREase and LACase activities were expressed in $kU.L^{-1}$.

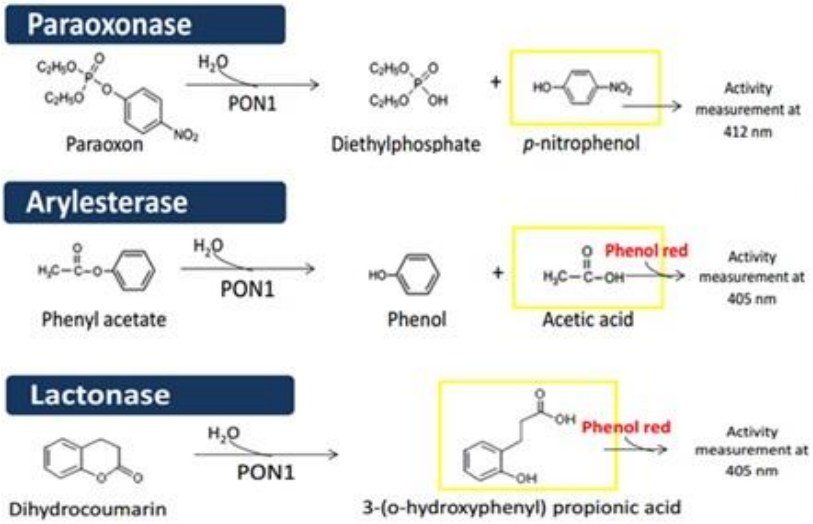

Figure 9 Analytical methodologies for measuring paraoxonase-1 activities. *Paraoxonase-1 (PON-1) enzyme has three activities identified to date. These activities can be spectrophotometrically quantified, employing different substrates and measuring the formation of specific products. PON-1 paraoxonase activity reflects the enzyme's ability to detoxify environmental toxicants; the substrate used for measuring this activity is paraoxon and the product monitored is p-nitrophenol (Batuca et al., 2008). PON-1 is also responsible for detoxifying lipid peroxides through its arylesterase activity, which can be measured using phenyl acetate as substrate and measuring acetic acid formation (Dias et al., 2014b). PON-1 also detoxifies homocysteine-thiolactone, through its lactonase activity, being this activity identified as the native activity of PON-1. For measuring lactonase activity, the substrate employed is dihydrocoumarin and the product of the enzymatic hydrolysis is 3-(o-hydroxyphenyl) propionic acid (Dias et al., 2014a). Adapted from Dias, 2013.*

1.3.3. Quantification of apolipoprotein A1 levels

ApoA1 plasma levels were determined in the RX Daytona analyser (*Randox Laboratories*, Northern Ireland, UK) by an immunoturbidimetric assay. ApoA1 levels were expressed as mg/dL.

1.3.4. Quantification of anti-high density lipoprotein and anti-apolipoprotein A1 IgG antibodies

Serum titres of anti-HDL and anti-ApoA1 IgG antibodies were quantified by in-house developed enzyme-linked immunosorbent assay (ELISA) methods, as previously described by Batuca and collaborators (Batuca et al., 2007, 2016). Briefly, 20 µg/mL HDL (*Sigma-Aldrich*, Portugal) or 10 µg/mL ApoA1 (*Sigma-Aldrich*) solutions were prepared in 70 % ethanol and then coated to a 96-well plate for 2 h at 37 ˚C. The plates were blocked at 37 ˚C with 100 µL/well of 1% bovine serum albumin (BSA) in 10 mM phosphate buffered saline (PBS) pH 7.4, for 1 h. After this period, the plates were washed 4X with PBS. Subsequently, samples, controls and standards were diluted in blocking buffer (1:100 dilution for quantifying anti-HDL antibodies and 1:200 dilution for quantifying anti-ApoA1 antibodies) and added to the plate for 1 h at 37 ˚C. The unbound antibodies were removed by repeating the washing step. Alkaline phosphatase conjugated anti-human IgG antibody (1:1000 dilution in blocking buffer) was then added to the plate for 1 h. After this period of incubation, plates were washed twice with PBS and BIC buffer. *P*-nitrophenyl phosphate diluted in BIC buffer was then added to the plate and incubated at 37 °C for 1 h. The absorbance was determined at 405 nm.

The assays were validated by the inclusion of internal quality control samples. The quantification of the anti-HDL IgG antibodies was presented as a percentage of the control, while the quantification of the anti-ApoA1 IgG antibodies was presented as µg/mL. The calibration curve for anti-ApoA1 IgG was prepared with six standards of ApoA1 monoclonal antibody (*Thermo Scientific*) with concentrations ranging from 0.001 to 0.04 µg/mL. Inter- and intra-plate variation was less than 10 %. Serum from 150 healthy volunteers was employed to assess a reference range for these auto-antibodies.

The cut-off for the upper normal levels of anti-HDL and anti-ApoA1 antibodies was set at the mean ± 3 times the standard deviation of the healthy controls. Therefore, samples were considered positive if the levels of anti-HDL and anti-ApoA1 were above 180.96 % and 0.274 µg/mL, respectively.

1.3.5. Statistical analysis

Statistical analysis was performed using GraphPad® Prism version 5.0 (*GraphPad Software Inc.*, La Jolla, CA, USA). Percentages, mean ± standard error of the mean (SEM) or median ± interquartile range (IQR) were used to describe the population. *Fisher's* test was employed for comparison between percentages while *Mann-Whitney U* test was performed for comparisons between medians. One-way analysis of variance (ANOVA) with *Bonferroni*'s multiple comparison post-hoc test was performed for comparing means between more than two groups. *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis was performed for comparing medians between more than two groups. Systemic exposure to NVP was evaluated in terms of absolute concentration while systemic exposure to NVP metabolites was evaluated as absolute concentrations and as the proportion of each compound in plasma, either adjusted or not by the body weight. Correlations between the end-points, NVP and its metabolites and the other collected parameters were obtained using *Spearman*'s or *Pearson's* test, whenever appropriate. Multivariable analysis was performed using IBM SPSS Statistics 23.0 (*IBM.,* Armonk, NY, USA). For the prospective analysis, the parameters of HDL functionality and lipid profile were normalised by the baseline values for each individual patient, and expressed in percentage from baseline. *One sample t* test and *Wilcoxon signed-rank* test were performed to compare prospective values with baseline. Differences between groups and correlations were considered significant if p value < 0.05.

1.4. Results

1.4.1. Prospective analysis

1.4.1.1. Characterisation of the patients

Eleven HIV-1-infected patients starting a NVP-based cART scheme were prospectively followed for up to 20 weeks. Demographic, anthropometric and clinical data for patients included in this prospective analysis is shown on Table 4. A total of 7 patients (64%) were of Caucasian ethnicity, 5 patients (45%) were women and the age of the participants ranged from 30 to 69 years old. Only 3 patients (27%) had previous experience on cART, while the remaining patients were on NVP-based cART as first antiretroviral therapy. Regarding cART backbone, 7 patients (64%) were treated with TDF plus FTC. On table 4, it is shown the levels of HIV-1 viral load, $CD4^+$ cell counts and liver function parameters for each patient, along the course of the study.

Table 4 Demographic, anthropometric and clinical data for patients included in the prospective study.

Patient	Ethnicity	Sex	Age (years)	Body weight (kg)	BMI (Kg/m²)	Prior cART experience	cART backbone	Visits	HIV-1 viral load (copies/mL)	CD4⁺ cell counts (cells/mm³)	ALT (U/L)	LDH (U/L)	ALKP (U/L)	GGT (U/L)	Time between sampling and last NVP intake (h)
1	NC	M	35	64	20	No	ABV + 3TC	Baseline	17089	280	73	314	211	275	-
								Week 12	BQL	352	74	209	199	621	12
2	C	M	55	65	23	No	ABV + 3TC	Baseline	142084	302	44	189	62	17	-
								Week 2	625	389	36	173	95	20	15
								Week 8	425	286	32	201	87	18	
								Week 16	127	672	23	181	77	22	15
3	NC	M	31	81	26	No	TDF + FTC	Baseline	49939	349	58	288	79	81	-
								Week 2	ND	ND	48	ND	ND	110	14
								Week 4	115	424	55	287	70	160	13
								Week 20	BQL	406	65	255	66	254	15
4	C	M	60	75	28	No	TDF + FTC	Baseline	39522	276	32	171	50	41	-
								Week 2	ND	ND	27	ND	ND	58	13
								Week 4	ND	230	25	202	56	96	15
								Week 12	145	224	27	176	64	119	13
5	C	F	69	49	23	Yes	TDF + FTC	Baseline	BQL	247	24	136	84	16	-
								Week 2	BQL	ND	ND	ND	ND	ND	16
								Week 4	BQL	ND	54	ND	ND	31	14
								Week 12	BQL	348	352	239	93	56	15

Patient	Ethnicity	Sex	Age (years)	Body weight (kg)	BMI (Kg/m²)	Prior cART experience	cART backbone	Visits	HIV-1 viral load (copies/mL)	CD4⁺ cell counts (cells/mm³)	ALT (U/L)	LDH (U/L)	ALKP (U/L)	GGT (U/L)	Time between sampling and last NVP intake (h)
6	C	F	57	69	29	No	TDF + FTC	Baseline	233983	42	112	218	85	186	-
								Week 2	ND	ND	96	ND	ND	190	13
								Week 4	3069	58	91	192	48	237	12
								Week 12	29801	114	69	219	57	181	11
7	NC	F	59	102	44	No	TDF + FTC	Baseline	20044	58	23	215	77	31	-
								Week 8	BQL	68	28	245	127	77	14
8	C	M	45	66	22	Yes	TDF + FTC	Baseline	BQL	709	27	181	102	130	-
								Week 2	BQL	ND	ND	ND	ND	ND	13
								Week 4	BQL	591	42	189	100	376	12
								Week 17	BQL	633	38	160	93	352	12
9	C	M	49	50	17	Yes	ABV + 3TC	Baseline	BQL	840	31	214	144	35	-
								Week 2	BQL	ND	61	ND	ND	ND	ND
10	NC	F	30	71	26	No	ABV + 3TC	Baseline	17089	280	38	ND	70	35	-
								Week 2	BQL	277	39	270	84	62	12
11	C	F	40	51	22	No	TDF + FTC	Baseline	ND	ND	32	149	48	16	-
								Week 12	BQL	788	39	156	55	38	15

Abbreviations: 3TC, lamivudine; ABV, abacavir; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; BQL, below quantification limit; C, Caucasian; cART, combined antiretroviral therapy; F, female; FTC, emtricitabine; GGT, gamma glutamyl-transferase; LDH, lactate dehydrogenase; M, male; NC, non-Caucasian; ND, non-determined; NVP, nevirapine; TDF, tenofovir disoproxil fumarate. Patient 9 interrupted NVP-based treatment due to skin rash. Missing weeks correspond to missed appointments or data unavailability.

1.4.1.2. Nevirapine treatment was associated to an overall improvement of high density lipoprotein-cholesterol levels and functionality

In order to investigate the effect of NVP in HDL quantity (HDL-cholesterol) and functionality (ApoA1, PON-1 activities, anti-HDL and anti-ApoA1 antibodies), a prospective analysis was conducted. In figure 10 is shown the temporal variation of study end-points, across the duration of the study (from baseline up to 8-20 weeks).

The exposure to 200 mg of NVP for 2 weeks allowed a decrease in anti-HDL levels (Figure 10, Panel B, *One sample t* test; mean ± SEM, -33 ± 11.8 % change from baseline, p= 0.039), without changes in HDL-cholesterol levels (Figure 10, Panel A, *One sample t* test; -3 ± 2.5 %, p> 0.05). This early decrease in anti-HDL antibodies contrasts with a delayed decrease in anti-ApoA1 antibodies (Figure 10, Panel D, *One sample t* test; week 2: 35 ± 24.8 %, p> 0.05; week 4: -18 ± 25.0 %, p> 0.05; week 8-20: -34 ± 9.7, p= 0.012). Also, the ApoA1 increase was a late event, reached when the steady-state concentration on 400 mg NVP was already attained (Figure 10, Panel C, *Wilcoxon signed-rank test*; median [IQR] week 8-20: 9 [3 - 22] %; p= 0.036). The most substantial changes were the decrease in anti-HDL (Figure 10, Panel B, *One sample t test*; week 4: -60 ± 17.9 %, p= 0.028), anti-ApoA1 (Figure 10, Panel D, *One sample t test*; week 8-20: -34 ± 9.7 %, p= 0.012), as well as the increase in HDL-cholesterol (Figure 10, Panel A, *One sample t test*; week 4: 32 ± 2.7 %, p= 0.0014) when patients were on 400 mg NVP.

PON-1 POase activity increased very slightly on week 8-20, not reaching statistical significance (Figure 10, Panel E, *One sample t test*; 9 ± 12.9 %, p> 0.05). PON-1 AREase activity gradually increased during the course of the study (Figure 10, Panel F, *One sample t test*; week 2: 5 ± 14.6 %; week 4: 23 ± 28.6; p> 0.05), reaching statistical significance on week 8-20 (76 ± 21.6%; p= 0.012). Relatively to PON-1 LACase activity, it was observed a decrease of this activity on week 2 and 4 (Figure 10, Panel G, *One sample t test*; week 2: -7 ± 9.1 %; week 4: -17 ± 12.5 %; p> 0.05) followed by a significant increase on week 8-20 (33 ± 9.1%; p< 0.05).

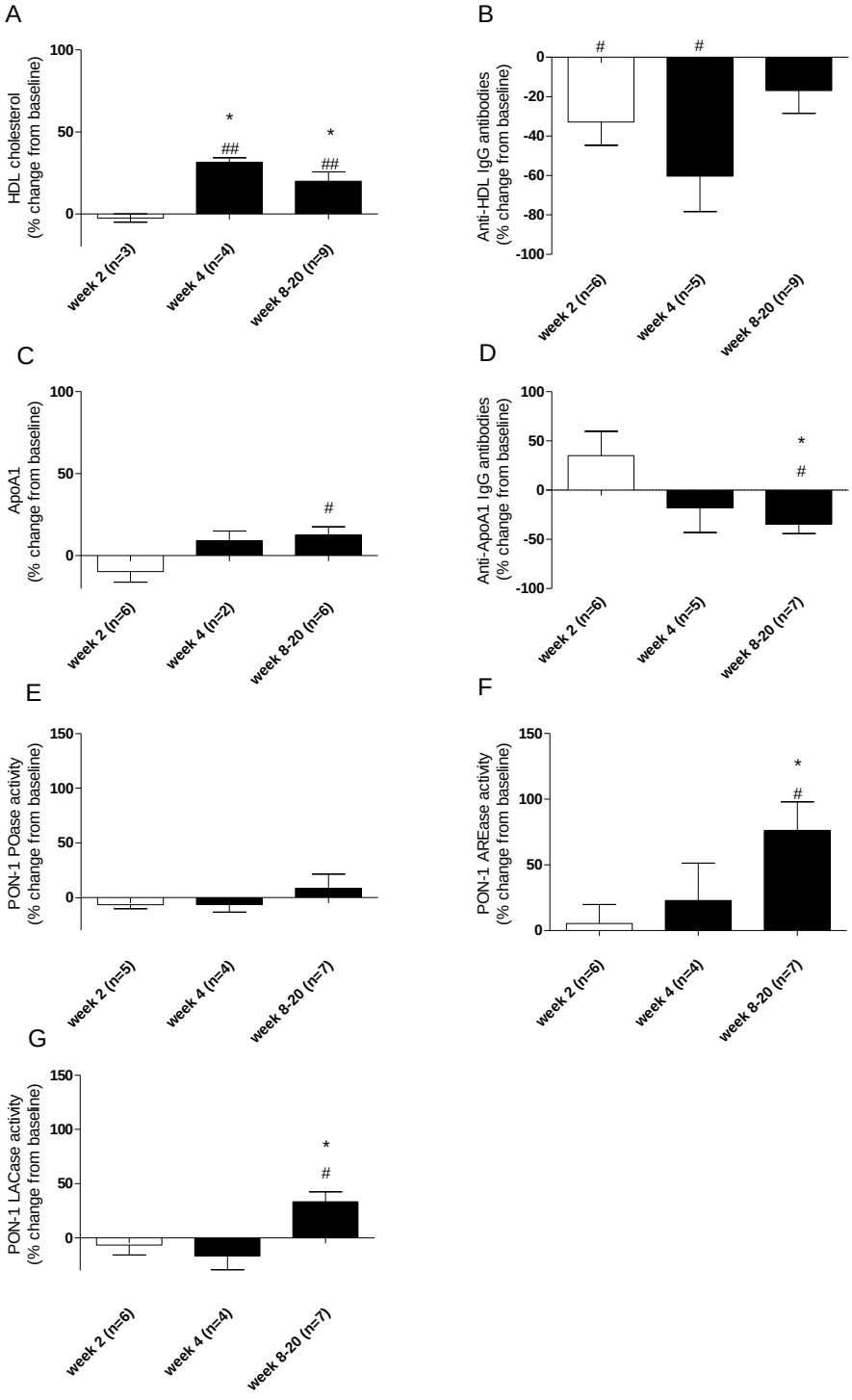

Figure 10 Effect of nevirapine treatment on high density lipoprotein-cholesterol levels and functionality. *Patients were prospectively followed during 2, 4 and 8 to 20 weeks after starting nevirapine (NVP)-based combined antiretroviral therapy (cART). Data regarding high density lipoprotein (HDL)-cholesterol (Panel A), anti-HDL IgG (Panel B), apolipoprotein A1 (ApoA1; Panel C), anti-ApoA1 IgG (Panel D) and paraoxonase (POase) activity (Panel E), arylesterase (AREase) activity (Panel F) and lactonase (LACase) activity (Panel G) of paraoxonase-1 (PON-1) is presented as % change from baseline. Kolmogorov-Smirnov test was employed to test normality. One sample t test was employed to compare means ± SEM to baseline (Panels A, B and D-G), while Wilcoxon signed-rank test was employed to compare medians [IQR] to baseline (Panel C). One-way ANOVA with Dunnett's multiple comparison post-test (Panels A, B and D-G) and Kruskal-Wallis test with Dunn's multiple comparison post-hoc analysis (Panel C) were employed to compare values between week 4 and 8-20 to week 2. Differences were considered significant if p< 0.05. (#) versus baseline; (*) versus week 2.*

1.4.2. Cross-sectional study

1.4.2.1. Characterisation of the study population

A total of 332 HIV-1 infected patients were included in the current study. One hundred and forty-six (146) of those patients were cART naïve (naïve group), which means that they did not meet the criteria for staring cART due to their early stage of infection; while 186 patients had been treated with a NVP-based cART scheme, receiving 400 mg of NVP once daily for at least 3 months (NVP-treated group). Demographic, anthropometric and clinical data for patients in the naïve group and in the NVP-treated group is presented in Table 5.

The percentage of patients of Caucasian ethnicity and the percentage of women between the naïve group and the NVP-treated group were not significantly different (*Fisher'*s test for comparison between proportions; $p > 0.05$). NVP-treated patients were older than naïve patients (*Mann-Whitney U* test for comparison between medians; $p < 0.0001$). There were no significant differences between groups regarding body weight, BMI or $CD4^+$ counts (*Mann-Whitney U* test; $p > 0.05$). For patients in NVP-treated group, the median time on cART was 8 years and the median time on NVP-based cART was 4 years. The most common cART backbones were TDF plus FTC (54% of patients on NVP-based cART) and abacavir (ABV) plus 3TC (40% of patients on NVP-based cART). Patients on NVP treatment had higher levels of liver function markers ALT, ALKP and GGT (*Mann-Whitney U* test*, p*< 0.001).

In order to characterise sex-related differences on the end-points, anthropometric and clinical parameters were analysed in accordance to sex and treatment status (Table 6). For instance, NVP-treated women presented lower body weight (Kg) than NVP-treated men, while there were no significant differences on body weight between naïve women and men (*Kruskal-Wallis* test with *Dunn'*s multiple comparison post-hoc analysis; $p < 0.01$ for NVP-treated male *versus* female). However, relatively to the BMI, the only sex-related differences observed were between naïve patients (*Kruskal-Wallis* test with *Dunn'*s multiple comparison post-hoc analysis; $p \leq 0.01$ for naïve male *versus* female).

Table 5 Demographic, anthropometric and clinical data for patients included in the cross-sectional study.

Parameters	Naïve patients (n=146)	NVP-treated patients (n=186)	*p* value
Percentage of Caucasians (%) [a]	53	55	NS
Percentage of females (%) [a]	48	53	NS
Age (years) [b]	38 [31 – 47]	46 [39 – 56]	< 0.0001
Body weight (kg) [b, c]	70 [58 – 81] (n=88)	70 [61 – 80] (n=184)	NS
BMI (kg/m^2) [b, c]	25 [21 – 29] (n=87)	25 [22 – 28] (n=183)	NS
HIV-1 viral load (copies/mL) [c]	21047 [4691 – 54318] (n=115)	BQL (n=173)	
CD4$^+$ cell counts (cells/mm^3) [b, c]	483 [388 – 642] (n=140)	519 [375 – 671] (n=166)	NS
Time on cART (years) [c]	–	8 [4 – 11] (n=121)	NA
Time on NVP (years) [c]	–	4 [2 – 8] (n=88)	NA
cART backbone (%)			
NVP + TDF + FTC	–	54	NA
NVP + ABV + 3TC	–	40	NA
NVP + AZT + 3TC	–	3	NA
NVP + ABV + FTC	–	2	NA
NVP + DDI + LPV/r	–	1	NA
Time between sampling and last NVP intake (h) [c]	–	13 [11 – 15] (n=52)	NA
ALT (U/L) [b, c]	24 [18 – 37] (n=94)	30 [25 – 45] (n=117)	0.0009
ALKP (U/L) [b, c]	71 [57 – 82] (n=88)	82 [69 – 124] (n=117)	< 0.0001
LDH (U/L) [b, c]	184 [162 – 215] (n=80)	196 [172 – 229] (n=117)	NS
GGT (U/L) [b, c]	25 [16 – 41] (n=88)	66 [41 – 116] (n=120)	< 0.0001

Abbreviations: 3TC, lamivudine; ABV, abacavir; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AZT, zidovudine; BMI, body mass index; BQL, below quantification limit; cART, combined antiretroviral therapy; DDI, didanosine; FTC, emtricitabine; GGT, gamma glutamyl-transferase; LDH, lactate dehydrogenase; LPV/r, lopinavir/ritonavir; NA, not applicable; NS, not significant; NVP, nevirapine; TDF, tenofovir disoproxil fumarate.

Data are shown as percentage or as median [IQR]. Differences were considered significant if $p < 0.05$. [a] *Fisher's* test; [b] *Mann-Whitney U* test, median [IQR]. [c] Missing values correspond to patients for whom anthropometric or clinical data were not available.

Naïve men presented higher viral load than naïve women (*Mann-Whitney U* test; $p \leq 0.01$), while both men and women under NVP treatment had undetectable viral loads. Despite these differences in terms of viral load between naïve men and women, no significant sex-related differences were observed on the immunological status (*Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; $p > 0.05$).

Table 6 Sex-related differences in anthropometric and clinical data for patients included in the cross-sectional study.

Parameters	Naïve patients (n=146)		NVP-treated patients (n=186)	
	Male (n=76)	**Female (n=70)**	**Male (n=88)**	**Female (n=98)**
Body weight (kg) [a, c]	68 [60 – 79] (n=37)	72 [56 – 87] (n=51)	74 [64 – 83] (n=87)	67 [58 –78] **§§** (n=97)
BMI (kg/m^2) [a, c]	23 [20 – 26] (n=36)	26 [22 – 32] ** (n=51)	25 [22 – 27] (n=86)	26 [23 – 29] (n=97)
HIV-1 viral load (copies/mL) [b, c]	30624 [8926 – 86280] (n=63)	9926 [614 – 26333] ** (n=52)	BQL (n=89)	BQL (n=93)
CD4$^+$ cell counts (cells/mm^3) [a, c]	459 [384 – 624] (n=73)	532 [389 – 669] (n=67)	526 [406 – 673] (n=76)	512 [357 – 663] (n=90)
ALT (U/L) [a, c]	26 [19 – 43] (n=57)	22 [16 – 32] (n=37)	34 [25 – 46] (n=55)	30 [23 – 39] **##** (n=62)
LDH (U/L) [a, c]	187 [172 – 215] (n=48)	172 [155 – 204] (n=32)	181 [170 – 215] (n=56)	206 [179 – 232] **##** (n=61)

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; BQL, below quantification limit; LDH, lactate dehydrogenase; NVP, nevirapine.

Data are shown as median [IQR]. Differences were considered significant if $p < 0.05$. [a] *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis. [b] *Mann-Whitney U* test. [c] Missing values correspond to patients for whom anthropometric or clinical data were not available. (**§**) *versus* NVP-treated male. (*) *versus* naïve male. (#) *versus* naïve female.

Regarding hepatic function tests, NVP-treated women showed higher levels of ALT (*Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; $p \leq 0.01$ for naïve female *versus* NVP-treated female) and higher levels of LDH ($p \leq 0.01$ for naïve female *versus* NVP-treated female) than naïve women. Differences between naïve male and NVP-treated male did not reach statistical significance. These observations relatively to

sex differences on hepatic function tests are consistent with female-predominant NVP-induced toxicity.

1.4.2.2. Patients on nevirapine treatment showed improved high density lipoprotein functionality

Data regarding patients' lipid profile and HDL functionality are presented in Table 7. Patients under NVP treatment presented higher levels of total cholesterol and HDL-cholesterol (*Mann-Whitney U* test; $p< 0.0001$ for both total cholesterol and HDL-cholesterol). LDL-cholesterol was also higher among NVP-treated patients, although to a lesser extent than total cholesterol and HDL-cholesterol ($p< 0.013$). No significant differences were observed for triglycerides levels ($p> 0.05$). Taken together, these observations support an increment of total cholesterol in the NVP-treated group mainly caused by increases in HDL-cholesterol levels. The levels of ApoA1, the precursor of HDL particles and main structural and functional protein present in HDL, were also increased in NVP-treated patients ($p< 0.0001$). Regarding PON-1 activities, NVP-treated patients presented higher POase and AREase activities ($p< 0.0001$ for both activities). PON-1 LACase activity was also higher among NVP-treated patients, although not reaching statistical significance ($p= 0.066$). Moreover, the levels of anti-HDL and anti-ApoA1 IgG, a measure of HDL and ApoA1 dysfunctionality, were lower among NVP-treated patients ($p= 0.0017$ for anti-HDL IgG; $p< 0.0001$ for anti-ApoA1 IgG). Regarding the quantification of these auto-antibodies, samples were considered positive if the levels of anti-HDL and anti-ApoA1 were above 180.96 % and 0.274 µg/mL, respectively. The percentage of patients with positive levels of anti-HDL and anti-ApoA1 was lower among NVP-treated patients (*Fisher's* test; $p= 0.046$ for anti-HDL; $p< 0.0001$ for anti-ApoA1).

Table 7 Lipid profile and high density lipoprotein functionality in HIV-infected naïve patients and patients under nevirapine treatment.

Parameters	Naïve patients (n=146)	NVP-treated patients (n=186)	*p* value
Total cholesterol (mg/dL)	167 [146 – 194] (n=127)	207 [179 – 231] (n=175)	< 0.0001
LDL-cholesterol (mg/dL)	109 [88 – 122] (n=122)	115 [97 – 138] (n=173)	0.013
HDL-cholesterol (mg/dL)	45 [35 – 56] (n=122)	61 [50 – 74] (n=174)	< 0.0001
Triglycerides (mg/dL)	92 [68 – 124] (n=127)	100 [71 – 147] (n=174)	NS
ApoA1 (mg/dL)	134 [121 – 155] (n=114)	152 [133 – 172] (n=181)	< 0.0001
PON-1 POase activity (U/L)	118 [52 – 194] (n=143)	210 [158 – 246] (n=181)	< 0.0001
PON-1 AREase activity (kU/L)	61 [27 – 95] (n=142)	118 [83 – 144] (n=180)	< 0.0001
PON-1 LACase activity (kU/L)	11 [9 – 14] (n=85)	12 [9 – 17] (n=176)	NS (*p*= 0.066)
Anti-HDL IgG antibodies (% positive control)	157 [112 – 224] (n=89)	113 [81 – 165] (n=90)	0.0017
Patients with positive titres of anti-HDL antibodies (%)	37	24	0.046
Anti-ApoA1 IgG antibodies (µg/mL)	0.24 [0.13 – 0.47] (n=36)	0.11 [0.06 – 0.20] (n=48)	< 0.0001
Patients with positive titres of anti-ApoA1 antibodies (%)	44	11	< 0.0001

Abbreviations: ApoA1, apolipoprotein A1; AREase, arylesterase; HDL, high density lipoprotein; IgG, immunoglobulin G; LACase, lactonase; LDL, low density lipoprotein; NS, not significant; NVP, nevirapine; POase, paraoxonase; PON-1, paraoxonase-1.

Data are shown as percentage or median [IQR]. Differences were considered significant if $p < 0.05$. *Mann-Whitney U* test and *Fisher's* test were performed as statistical tests. Missing values correspond to patients for whom quantification of lipid profile or parameters of HDL functionality were not performed.

1.4.2.3. Sex and nevirapine treatment affect high density lipoprotein functionality

In order to clarify if the effect of NVP treatment in HDL functionality was sex-dependent, data were analysed considering four groups of patients according to treatment status – naïve or NVP-treated – and sex (Figure 11). Sex-related differences were observed for naïve patients, with women presenting higher levels of HDL-cholesterol (Figure 11, Panel A, *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; naïve male: 38 [32 – 46] (n=66); naïve female: 56 [45 – 63] (n=56); $p \leq 0.001$), ApoA1 (Figure 11, Panel C, One-way ANOVA with *Bonferroni*'s multiple comparison post-hoc test; naïve male: 128 ± 3.4 (n=62); naïve female: 146 ± 2.7 (n=51); $p \leq 0.01$) and PON-1 AREase activity (Figure 11, Panel F, *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; naïve male: 41 [21 – 70] (n=75); naïve female: 86 [45 – 106] (n=67); $p \leq 0.001$).

Approximately the same pattern was observed for NVP-treated patients, with women presenting higher levels of HDL-cholesterol (Figure 11, Panel A, NVP-treated male: 55 [46 – 66] (n=79); NVP-treated female: 67 [55 – 83] (n=95); $p < 0.001$) and higher ApoA1 levels (Figure 11, Panel C, NVP-treated male: 145 ± 3.7 (n=85); NVP-treated female: 159 ± 3.2 (n=92); $p < 0.01$). However, PON-1 AREase activity was similar between NVP-treated males and females (Figure 11, Panel F, NVP-treated male: 115 [73 – 141] (n=83); NVP-treated female: 119 [88 – 147] (n=97); $p \geq 0.05$). NVP-treated female was the group presenting a more favourable lipid profile, taken together these observations support an interaction between sex and NVP treatment in the modulation of HDL.

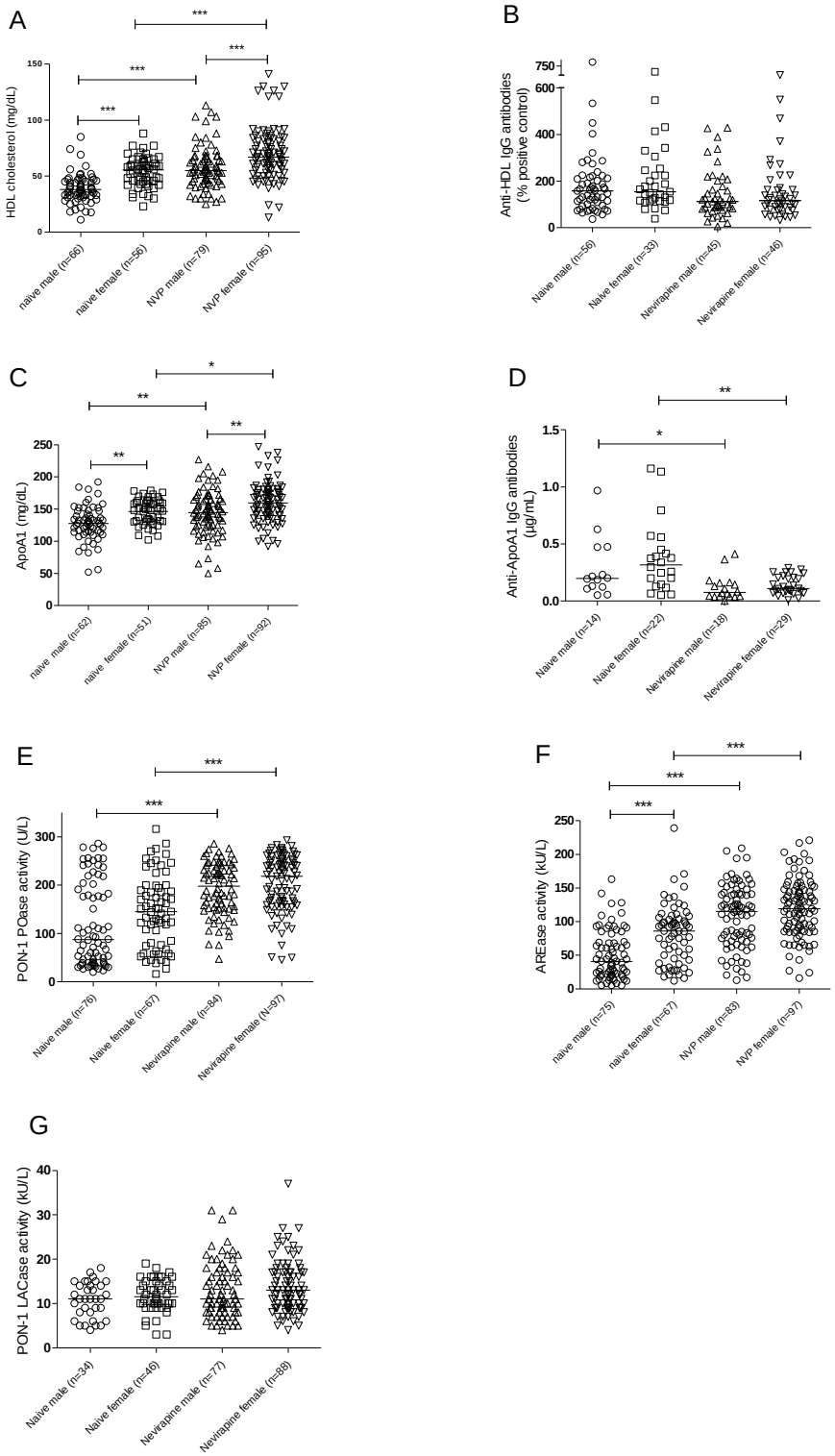

Figure 11 Sex-related and nevirapine-related differences on lipid profile and high density lipoprotein functionality. *Patients were divided into 4 groups according to treatment status (naïve or nevirapine (NVP)-treated) and sex. Data regarding high density lipoprotein (HDL)-cholesterol (Panel A), anti-HDL IgG antibodies (Panel B), anti-apolipoprotein A1 (ApoA1) IgG antibodies (Panel D), paraoxonase (POase) activity (Panel E), arylesterase (AREase) activity (Panel F) and lactonase (LACase) activity (Panel G) of paraoxonase-1 (PON-1) enzyme are presented as median [IQR], while data on ApoA1 (Panel C) are presented as mean ± SEM. Kruskal-Wallis test with Dunn's multiple comparison post-hoc analysis was performed for comparison between medians on Panels A, B and D-G, while One-way ANOVA with Bonferroni's multiple comparison post-hoc test was performed for comparison between means on Panel C. Differences were considered significant if p< 0.05. *p< 0.05; ** p≤ 0.01; *** p≤ 0.001. Missing values correspond to patients for whom quantification of lipid profile or parameters of HDL functionality were not performed.*

1.4.2.4. Nevirapine effect on high density lipoprotein functionality is a drug-specific effect

In order to investigate whether the improvement on HDL functionality was intrinsically related to NVP or a consequence of the suppression of HIV replication, and therefore not a drug-specific effect, we compared two of the end-points analysed in the current study – PON-1 POase activity and anti-HDL antibodies – in naïve patients and in patients on different cART schemes: NVP-, EFV- and PIs-based treatment.

A total of 54 HIV-infected patients on EFV-based cART (72% of Caucasian ethnicity, 44% women, age ranging from 20 to 72 years) and 63 patients on PIs-based cART (71% of Caucasian ethnicity, 28% women, age ranging from 25 to 83 years) were included. NVP-treated patients presented higher PON-1 POase activity than any other group (Figure 12, Panel A, *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; NVP: 210 [158 - 246] n=181; naïve: 118 [52 - 194] n=143, NVP *vs*. naïve $p< 0.001$; EFV: 143 [89 - 225] n=30, NVP *vs*. EFV $p< 0.05$; PIs: 102 [58 - 195] n=44, NVP *vs*. PIs $p< 0.001$). Likewise, NVP-treated patients presented lower levels of anti-HDL antibodies than any other group (Figure 12, Panel B, *Kruskal-Wallis* test with *Dunn*'s multiple comparison post-hoc analysis; NVP: 113 [81 - 165] n=90; naïve: 157 [112 - 224] n=89, NVP *vs*. naïve $p< 0.01$; EFV: 143 [121 - 186] n=54; NVP *vs*. EFV $p< 0.05$; PIs: 144 [113 - 205] n=63; NVP *vs*. PIs $p< 0.05$).

Additionally, we evaluated the relations between the viral load in naïve patients and the study end-points. We found a negative correlation between the viral load and the levels of HDL-cholesterol (*Pearson's* test; r= -0.24; $p= 0.018$; n=96). HIV-1 viral load was not correlated with ApoA1 (*Pearson's* test; r= -0.05; $p= 0.63$; n=90), anti-HDL antibodies (*Spearman*'s test; r= 0.07; $p= 0.52$; n=77), anti-ApoA1 antibodies (*Spearman*'s test; r= 0.04; $p= 0.85$; n=27) or PON-1 activities (*Spearman*'s test; POase: r= 0.08; $p= 0.41$; n=112; AREase: r= -0.09; $p= 0.31$; n=111; LACase: r= -0.12; $p= 0.36$; n=61). Taken together, these observations support that improvement of HDL functionality induced by NVP treatment is a drug-specific effect, although we cannot exclude the suppression of viral replication as a factor contributing for HDL modulation, particularly in terms of HDL-cholesterol levels.

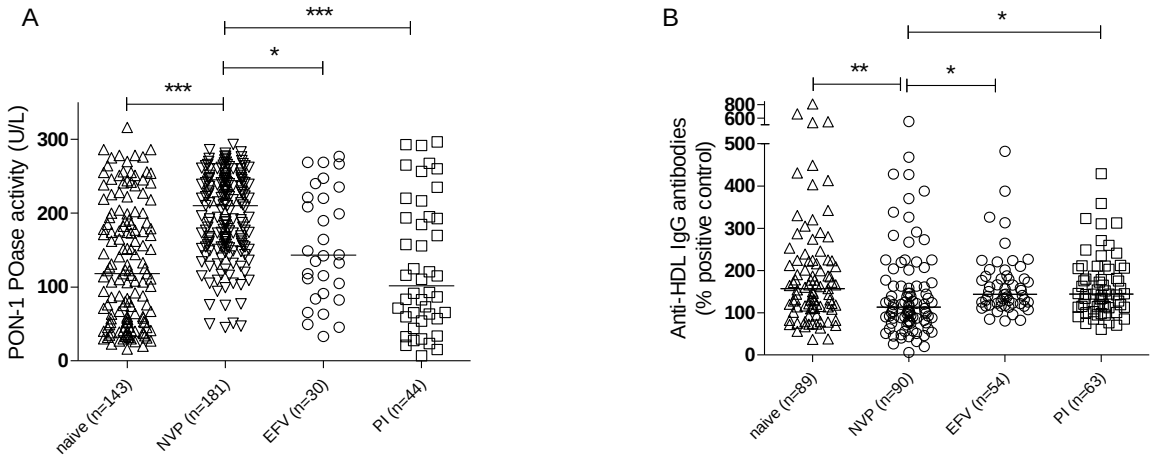

Figure 12 High density lipoprotein functionality in HIV-infected patients treated with different antiretroviral therapies. *Patients were divided into four groups according to treatment status: naïve patients, patients treated with nevirapine (NVP), efavirenz (EFV) or protease inhibitors (PI). Data regarding paraoxonase (POase) activity of paraoxonase-1 (PON-1) enzyme (Panel A) and anti-high density lipoprotein (HDL) antibodies (Panel B) are presented as median [IQR]. Kruskal-Wallis test with Dunn's multiple comparison post-hoc analysis was employed as statistical test. Differences were considered significant if p< 0.05. *p< 0.05; ** p≤ 0.01; *** p≤ 0.001.*

1.4.2.5. Nevirapine biotransformation seems to influence, at least to some extent, the functionality of high density lipoprotein particles

Multivariable analysis was performed for NVP-treated patients in order to clarify the factors associated with the study end-points in these patients, particularly the influence of NVP biotransformation on HDL functionality. The plasma levels of NVP and its metabolites are presented on Table 8.

PON-1 LACase activity was associated with AREase activity (Multivariable analysis; B: 3.9; 95% CI [3.1 – 4.8]; $p< 0.001$). Additionally, the proportion of 3-OH-NVP (B: -5.6 ± 1.9; $p= 0.009$), the proportion of 12-OH-NVP (B: -3.5 ± 1.1; $p= 0.007$) and the proportion of 2-OH-NVP adjusted by body weight (B: -2.7 ± 0.8; $p= 0.005$) were negatively associated with PON-1 LACase activity. The proportion of 2-OH-NVP in plasma was negatively associated with anti-HDL antibodies (B: -14.4; 95% CI [-27.6 – -1.3]; $p= 0.03$).

Additionally, in the multivariable analysis, NVP concentration was associated with the plasma levels of 2-OH-NVP (B: 8.2 ± 3.8; $p= 0.03$), 12-OH-NVP (B: 2.8 ± 0.8; $p< 0.001$) and particularly with the plasma levels of 3-OH-NVP (B=62.2 ± 16.8; $p< 0.001$). However, NVP plasma levels were not associated with the proportions of the metabolites ($p> 0.05$ for

proportions of 2-, 12- and 3-OH-NVP, either or not adjusted by the body weight). Moreover, the proportion of 3-OH-NVP was negatively associated with the proportions of 2-OH-NVP (B: -1 ± 0.006; $p< 0.001$) and 12-OH-NVP (B: -1 ± 0.005; $p< 0.001$).

Table 8 Quantification of nevirapine metabolite profile and sex-related differences in phase I biotransformation.

	NVP-treated male (n=88)	**CV (%)**	**NVP-treated female (n=98)**	**CV (%)**	***p* value**
Analytes (ng. mL^{-1})					
NVP	4628 [3183 – 5489] (n=60)	38	4233 [3445 – 5405] (n=63)	42	NS
2-OH-NVP	57 [33 – 104] (n=40)	68	46 [27 – 66] (n=44)	55	NS
3-OH-NVP	24 [17 – 31] (n=51)	35	25 [18 – 36] (n=57)	57	NS
12-OH-NVP	371 [285 – 543] (n=60)	47	375 [285 – 532] (n=64)	64	NS
Analytes adjusted by body weight (ng. mL^{-1}. Kg^{-1})					
NVP	61 [43 – 75] (n=57)	37	67 [50 – 89] (n=60)	42	0.039
2-OH-NVP	0.81 [0.44 – 1.44] (n=40)	75	0.76 [0.37 – 1.02] (n=44)	64	NS
3-OH-NVP	0.30 [0.23 – 0.44] (n=50)	40	0.41 [0.25 – 0.61] (n=57)	66	0.039
12-OH-NVP	5 [4 – 8] (n=59)	44	6 [4 – 8] (n=61)	61	NS
Proportions of metabolites in plasma (%)					
2-OH-NVP	11.2 [7.3 – 16.6] (n=40)	61	8.9 [6.6 – 14.0] (n=43)	63	NS
3-OH-NVP	4.6 [3.4 - 6.3] (n=49)	42	6.1 [4.1 – 8.0] (n=57)	48	0.027
12-OH-NVP	87 [80 – 94] (n=61)	11	88 [82 – 94] (n=60)	8	NS
Proportions of metabolites in plasma, adjusted by body weight (%. Kg^{-1})					
2-OH-NVP	0.17 [0.10 – 0.24] (n=40)	63	0.13 [0.09 – 0.21] (n=43)	67	NS
3-OH-NVP	0.06 [0.05 – 0.09] (n=51)	47	0.09 [0.05 – 0.13] (n=55)	52	0.024
12-OH-NVP	1.2 [1.0 – 1.3] (n=58)	17	1.3 [1.1 – 1.5] (n=62)	24	0.013

Abbreviations: 2-OH-NVP, 2-hydroxy-nevirapine; 3-OH-NVP, 3-hydroxy-nevirapine; 12-OH-NVP, 12-hydroxy-nevirapine; CV, coefficient of variation; NS, not significant; NVP, nevirapine. Data are shown as median [IQR] and percentage. The statistical test employed was *Mann-Whitney* U test. Differences were considered significant if $p< 0.05$. Missing values correspond to patients for whom the metabolite concentration was not quantified or was below the lower limit of quantification for the method.

1.5. Discussion

To the best of our knowledge this is the first study that investigated the intricate relations between NVP-induced modulation of HDL functionality and NVP biotransformation. The results reported herein support that NVP treatment has an overall positive impact in the modulation of several aspects of HDL functionality. Moreover, this study shows for the first time that NVP biotransformation may impact the modulation of HDL quality.

In the prospective study it was demonstrated an improvement in HDL functionality among patients starting NVP-based cART. However, the modulation of different aspects of HDL function varied along time (Figure 13): the decrease in anti-HDL antibodies was the earliest event, followed by a mid-late increase in HDL-cholesterol. The delayed events observed in the prospective analysis were the decrease in anti-ApoA1 antibodies and the increases in ApoA1 and PON-1 AREase and LACase activities. Interestingly, it was reported that NVP metabolite profile changes over time, from the administration of 200 mg NVP once daily to the steady-state 400 mg NVP dose (Fan-Havard et al., 2013). In fact, the 2-OH-NVP metabolite is predominant only in an early phase, while in the steady-state there is a decrease in the levels of 2-OH-NVP and the concomitant increase in 3-OH-NVP levels (Fan-Havard et al., 2013). This switch in NVP phase I metabolites is in accordance with the metabolite profile reported in our study, in which NVP plasma concentration in the steady-state is strongly associated with high levels of 3-OH-NVP (*cf.* Section 1.4.2.5). Moreover, this early predominance of 2-OH-NVP and the early decrease in the levels of anti-HDL antibodies, observed in the prospective study, are also consistent with our observation of the 2-OH-NVP proportion in plasma being associated with low levels of anti-HDL antibodies (*cf.* Section 1.4.2.5), suggesting that NVP biotransformation might affect the modulation of HDL functionality.

The switch in NVP metabolite profile towards higher formation of 3-OH-NVP might be mediated through CAR activation. It is known that NVP is a CAR agonist (Faucette et al., 2006), being able to induce CYP2B6 through CAR activation (Erickson et al., 1999; Faucette et al., 2006) and CYP2B6 is the only CYP450 isoform involved in the formation of 3-OH-NVP (Erickson et al., 1999).

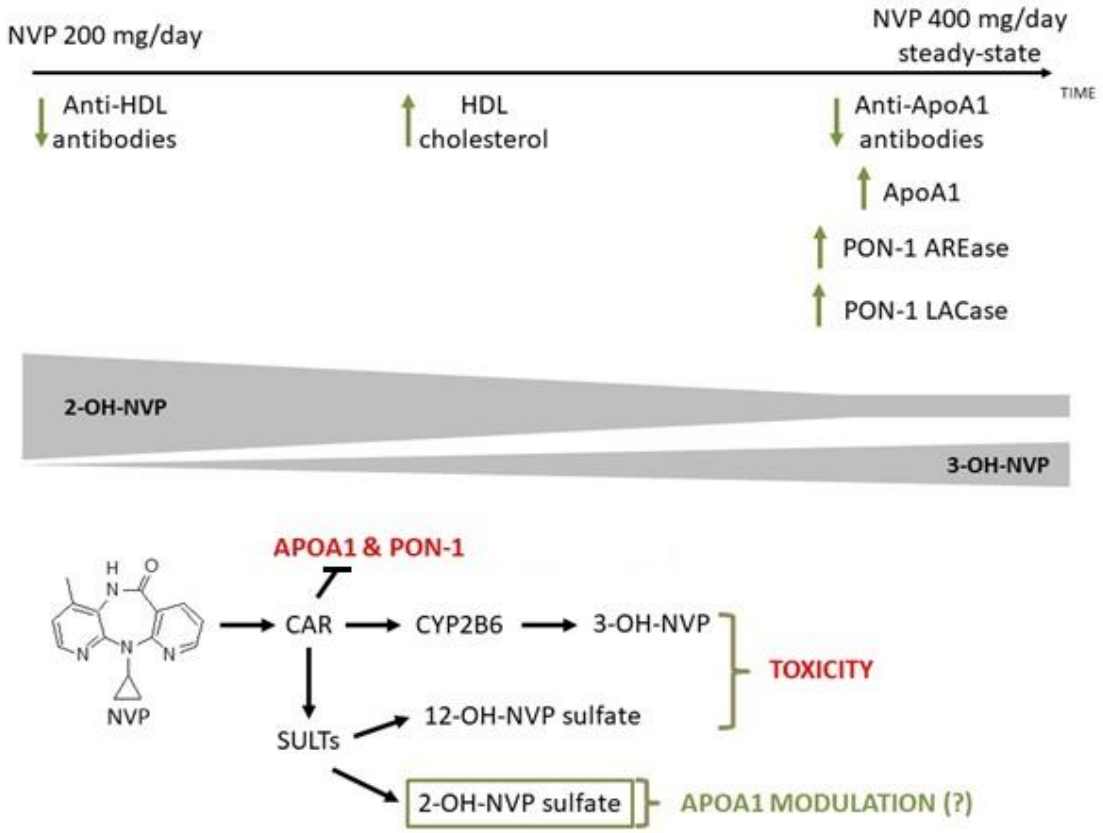

Figure 13 Schematic representation of the interplay between nevirapine metabolism and high density lipoprotein modulation. *The modulation of different aspects of high density lipoprotein (HDL) functionality in patients starting nevirapine (NVP) treatment varies along time. The decrease in anti-HDL antibodies was the earliest event observed, followed by increases in HDL-cholesterol. The later events were the decrease in anti-apolipoprotein A1 (ApoA1) antibodies and the increases in ApoA1, arylesterase (AREase) and lactonase (LACase) activities of paraoxonase-1 (PON-1) enzyme. NVP metabolite profile also changes over time, from the administration of 200 mg NVP once daily to the steady-state 400 mg NVP dose. The 2-hydroxy-nevirapine (2-OH-NVP) metabolite is predominant in an early phase, while in the steady-state there is a decrease in the levels of 2-OH-NVP and the concomitant increase in 3-hydroxy-nevirapine (3-OH-NVP) levels (Fan-Havard et al., 2013). This early predominance of 2-OH-NVP is coincident with the early decrease in the levels of anti-HDL antibodies. The switch in NVP metabolite profile might be mediated via constitutive androstane receptor (CAR) activation. It is known that NVP induces CYP2B6, the only CYP450 isoform involved in 3-OH-NVP formation, through CAR activation (Erickson et al., 1999; Faucette et al., 2006). Moreover, CAR activation promotes the induction of several hepatic sulfotransferases (SULTs), then promoting the sulfoconjugation of NVP metabolites (Alnouti and Klaassen, 2008; Sharma et al., 2013; Pinheiro et al., 2017), therefore decreasing the plasma levels of unconjugated 2-OH-NVP. This assumption implies that NVP could not be directly responsible for the modulation of HDL functionality, as CAR activation leads to decreased levels of ApoA1 and PON-1 (Masson et al., 2008; Cheng and Klaassen, 2012; Naik et al., 2013). ApoA1 and PON-1 modulation probably occur only when there is enough accumulation of a NVP metabolite(s), with a more lipid-friendly profile than NVP.*

Furthermore, CAR activation promotes the induction of several hepatic SULTs (Alnouti and Klaassen, 2008), that can promote the sulfoconjugation of 2-OH-NVP (Pinheiro et al., 2017) and 12-OH-NVP (Sharma et al., 2013; Pinheiro et al., 2017), therefore decreasing the plasma levels of free unconjugated metabolites. As NVP is also an inducer of CYP3A4, besides CYP2B6, via CAR activation (Faucette et al., 2006), increased

formation of 2-OH-NVP is expected as well, through CYP3A4 pathway (Erickson et al., 1999). Therefore, it is probable that increased sulfoconjugation of 2-OH-NVP is underlying the decrease in the levels of this metabolite, as reported by Fan-Havard and collaborators (2013). Thus, the 3-OH-NVP increase and the 2-OH-NVP decrease in the steady-state seems to be consistent with CAR activation induced by chronic NVP treatment (Figure 13). However, NVP might not be directly responsible for the modulation of HDL functionality, since CAR activation leads to decreased transcription of ApoA1 (Masson et al., 2008; Naik et al., 2013) and PON-1 (Cheng and Klaassen, 2012) and decreased levels of HDL-cholesterol (Masson et al., 2008; Naik et al., 2013). This assumption points towards a later modulatory effect on HDL, when there is enough accumulation of a NVP metabolite(s), with a more lipid-friendly profile than NVP (Figure 13).

In accordance with the observations of the prospective study, patients under chronic NVP treatment showed a better lipid profile and HDL function in the cross-sectional analysis. NVP treatment was associated with higher levels of HDL-cholesterol and ApoA1, lower levels of anti-HDL and anti-ApoA1 antibodies, as well as higher PON-1 activities. These observations are particularly relevant considering the impact of HDL-cholesterol levels and HDL function on atheroprotection. For instance, it was classically demonstrated the association between HDL-cholesterol levels and the prevention of cardiovascular events, in which an increase of only 1 mg/dL in HDL-cholesterol corresponds to a decrease of 2-3% in the risk of coronary heart disease, depending on the sex of the patient (Gordon et al., 1989). Moreover, the upregulation of ApoA1 was recently recognised as the most promising approach to improve HDL function and for the long-term prevention of atherosclerosis (Gadkar et al., 2016). Regarding the functionality of HDL particles, it is important to consider the role of auto-antibodies towards HDL and HDL components. For instance, the generation of anti-HDL and anti-ApoA1 antibodies has been recognised as strongly pro-atherogenic and pro-inflammatory, as these antibodies are able to induce inflammatory pathways upon interaction with Toll-like receptor (TLR)2 and TLR4 (Chistiakov et al., 2016). In fact, the generation of functional non-immunogenic ApoA1 molecules, able to act as a lipid-acceptor protein, and therefore being able to effectively promote reverse cholesterol

transport and consequently increase HDL-cholesterol levels, is regarded as an important atheroprotective factor (Schreier et al., 1999; Jian et al., 2013; Huang et al., 2014; Li et al., 2014; Sung et al., 2015; Rhee et al., 2017).

Interestingly, when considering separated groups of patients, according to their sex and treatment status, the sex-dependent differences were clear. Women on NVP treatment presented the highest blood levels of both HDL-cholesterol and ApoA1. Sex dimorphism in the lipoprotein levels, particularly in HDL and ApoA1 but also extending to other lipid parameters has been reported for non-HIV infected individuals (Ordovas et al., 2002). This sex-dependent effect can be mechanistically explained by the upregulation of hepatic ApoA1 promoted by the activation of oestrogen receptor α (ERα)-related intracellular pathways in women (Lamon-Fava et al., 1999; Kajinami et al., 2005). Another 1st line NNRTI, EFV, directly binds to ERα (Sikora et al., 2010). EFV and NVP share many pharmacological features: both NNRTIs are potent CAR activators (Faucette et al., 2006; Byakika-Kibwika et al., 2012) and both drugs are able to increase HDL-cholesterol levels and improve HDL antioxidant properties (Pereira et al., 2006, 2009). Interestingly, the induction of hepatic CYP3A4 and CYP2B6 can also be achieved through ERα-mediated signalling (Choi et al., 2013). However, if NVP or a NVP metabolite could activate ERα in the liver remains unknown. Nevertheless, ERα-mediated hepatic signalling is a relevant mechanism underlying the effect of sex on HDL and ApoA1 levels (Lamon-Fava et al., 1999; Kajinami et al., 2005) and cross-talk between CAR and ERα must be considered (Kawamoto et al., 2000; Min et al., 2002). Additionally, sex differences in biotransformation, such as the female-predominant expression of hepatic SULTs and PAPS (Alnouti and Klaassen, 2006, 2011), can also contribute to this effect in NVP-treated women.

In addition to sex, other factors might have a relevant impact on patient's lipid profile and HDL. For instance, the HIV viral load can contribute to lower levels of HDL-cholesterol (El-Sadr et al., 2005). Accordingly, in the current work HIV viral load was negatively associated with levels of HDL-cholesterol. Sex differences were also observed for the HIV-1 viral load in cART naïve patients, being the plasma levels of HIV-1 RNA significantly higher among men. This observation is in accordance with other studies reporting lower viral loads in female patients at earlier stages of HIV infection (Gandhi

et al., 2002; Donnelly et al., 2005). It is recognised the deleterious effect of the HIV infection on the lipid profile of seropositive individuals, and its association with higher levels of triglycerides and lower levels of both HDL- and LDL-cholesterol (El-Sadr et al., 2005). This HIV-associated pro-atherogenic profile is probably caused by virus-induced immune activation and chronic inflammation (Hsue et al., 2009; Bryant et al., 2016), being the C3 component of the complement cascade a possible player in this effect (Bryant et al., 2016). In the current study HIV-1 viral load was not correlated with the end-points reflecting HDL functionality (ApoA1, anti-HDL and anti-ApoA1 antibodies and PON-1 activities). Importantly, the naïve patients included in our study were left untreated for HIV due to their early stage of infection, immunological status (median $CD4^+$ cells/mm^3 was 483) and absence of HIV/AIDS-related symptoms. It is described that more dramatic changes in lipid profile and HDL-cholesterol levels are associated with disease progression (Ogunro et al., 2008). Also, increased formation of anti-ApoA1 antibodies is more frequently observed in advanced stages of HIV infection (Satta et al., 2017). Furthermore, here we have demonstrated that NVP-induced modulation of PON-1 and anti-HDL antibodies is a drug-specific effect, not observed on patients treated with PIs or EFV (*cf*. Section 1.4.2.4). Accordingly, in a study conducted with uninfected newborns receiving NVP treatment for HIV prophylaxis, it was demonstrated that NVP modulatory effect on HDL-cholesterol and ApoA1 is independent of viral replication control (Sankatsing et al., 2007); likewise NVP maintains its lipid-friendly and ApoA1 booster properties in HIV-1-infected adults with suppressed viral load (Franssen et al., 2009).

Moreover, considering that in our cross-sectional study NVP-treated patients were significantly older than naïve patients, the results presented herein are even more significant from a clinical point of view, as NVP treatment was associated with a positive modulation of HDL in an older population.

Besides HDL-cholesterol and ApoA1, the pharmacological modulation of PON-1 activities can also reduce the atherosclerotic risk (Sena et al., 2013). PON-1 is a circulating enzyme that is associated to HDL particles; this enzyme presents essential anti-atherosclerotic and antioxidant properties (Soran et al., 2015; Chistiakov et al., 2016; Sun et al., 2017). While PON-1 POase activity is mainly involved in xenobiotics detoxification (Costa et al.,

2005; Valiyaveettil et al., 2012; Bigley and Raushel, 2013; Karlsson et al., 2015), its AREase activity is involved in detoxifying lipid peroxides and limiting the levels of such peroxides in the atherosclerotic lesion (Mackness et al., 1993; Aviram et al., 2000; Mehdi and Rizvi, 2012; Kunutsor et al., 2016). On its turn, PON-1 LACase activity, the native activity of this enzyme, is involved in homocysteine-thiolactone detoxification, avoiding protein *N*-homocysteinylation (Jakubowski, 2000; Perła-Kaján and Jakubowski, 2012), that has been linked to cardiovascular and degenerative diseases (Jakubowski, 2006; Lakshman et al., 2006; Kamila et al., 2012; Khodadadi et al., 2012; Sharma et al., 2015). Additionally, PON-1 LACase activity can hydrolyse oxidised metabolites of polyunsaturated fatty acids and acyl homoserine lactones (Draganov et al., 2005; Manolescu, 2013). In the current study, the proportions of 2-, 3- and 12-OH-NVP were associated with lower PON-1 LACase activity (*cf.* Section 1.4.2.5), which can be a reason for lower LACase activity among patients chronically treated with NVP, compared to the much higher POase and AREase activities (*cf*. Table 7). This observation might result from an *in vivo* interaction between metabolites or other factors, such as inflammation, oxidative stress or viral infection, that can be deleterious to this specific activity (Nguyen et al., 2009). Besides, PON-1 activities can be affected by many other factors, such as polymorphisms and ethnicity (Draganov and La Du, 2004; Davis et al., 2009; Pereira et al., 2009; Woudberg et al., 2016), exposure to xenobiotics (Pereira et al., 2009; Kim et al., 2013; Pastryk et al., 2016), HIV infection (Daminelli et al., 2008; Dias et al., 2014a; Maselli et al., 2014), sex and circulating levels of sex hormones (Sutherland et al., 2001; Žitňanová et al., 2011; Schrader et al., 2012) and also by age (Rainwater et al., 2009). For instance, uncontrolled HIV-1 infection has been associated with lower PON-1 POase (Daminelli et al., 2008; Maselli et al., 2014) and LACase activities (Dias et al., 2014a). However, HIV treatment with some PIs (*eg*. ritonavir, atazanavir, saquinavir) can have an extremely deleterious impact on PON-1 activities, that can even surpass the effect of HIV infection itself (Daminelli et al., 2008; Pastryk et al., 2016), while HIV treatment with the NNRTI EFV can positively modulate PON-1 (Pereira et al., 2009). Regarding the effect of sex hormones, the activation of oestrogen receptors seems to partially mediate PON-1 upregulation in hepatic cells (Schrader et al., 2012) and hormone-replacement therapy with conjugated oestrogen and medroxyprogesterone acetate significantly increased PON-1 AREase activity in post-menopausal women (Sutherland et al., 2001) while

perimenopausal status was associated with decreased AREase and LACase activities (Žitňanová et al., 2011). Thus, a possible explanation for the sex differences on PON-1 AREase activity among naïve patients, observed in the present study, might be the combined effect of discrepant viral loads observed between men and women and the impact of sex hormones. Ethnicity is another factor that can impact PON-1 activities at some extent, with Caucasian individuals presenting lower PON-1 POase activity (Pereira et al., 2009; Woudberg et al., 2016) and slightly higher PON-1 AREase activity (Davis et al., 2009) than Black race individuals. In spite of this evidence, race is probably not a factor affecting the observations presented in the current work, since there were no differences in the ethnic composition of naïve and NVP-treated groups in the cross-sectional study. Age is reported to have a negative impact in all three PON-1 activities (Rainwater et al., 2009), therefore, considering the older age of NVP-treated patients in the present study, the modulation of PON-1 activities by chronic NVP treatment is even more relevant. Moreover, the formation of auto-antibodies against the HDL particle or against HDL components is also another factor that can negatively affect PON-1 activities and HDL antioxidant properties, as reported for the anti-HDL and anti-ApoA1 antibodies on PON-1 POase activity (Batuca et al., 2007, 2016).

In conclusion, we have demonstrated that NVP treatment is able to beneficially modulate several aspects of HDL functionality and that NVP biotransformation seems to be a factor in this NVP-induced effect. Here we provide evidence that NVP can be a suitable starting point for the development of an HDL booster molecule. The main downside of NVP is its association with idiosyncratic toxic reactions, which is mainly dependent on SULTs-mediated bioactivation of 12-OH-NVP (Antunes et al., 2008, 2010a, 2010b; Caixas et al., 2012; Sharma et al., 2013; Marinho et al., 2014c; Pinheiro et al., 2017), although other metabolic pathways may be involved in NVP toxicity (Antunes et al., 2011). Even though, NVP metabolites can be useful for the rational design of new HDL-booster and ApoA1-booster molecules with an improved toxicity profile.

CHAPTER 2. NEVIRAPINE MODULATION OF PARAOXONASE-1 IN THE LIVER: AN *IN VITRO* THREE MODEL APPROACH

Chapter 2

Nevirapine modulation of paraoxonase-1 in the liver: an *in vitro* three model approach

2.1. Summary

NVP is associated with severe hepatotoxicity, through the formation of reactive metabolites. PON-1 is a promiscuous enzyme involved in the metabolism of xeno- and endobiotics and proposed as a biomarker of hepatotoxicity. The aim of this work was to explore the effects of NVP and its phase I metabolites, 2-OH-NVP and 12-OH-NVP, on PON-1 activities. 2D and 3D primary cultures of rat hepatocytes, and also HepG2 2D cell cultures, were exposed to NVP, 2-OH-NVP and 12-OH-NVP. The POase, AREase and LACase activities of PON-1 were quantified. Effects of NVP and its metabolites were only observed in the 3D cell model. Both NVP and 12-OH-NVP increased POase ($p< 0.05$, $p< 0.01$) and LACase activities ($p< 0.05$, $p< 0.001$). The AREase activity was increased only upon 12-OH-NVP exposure ($p< 0.01$). These modulatory effects were observed at 300µM concentrations of NVP and 12-OH-NVP. The formation of 12-OH-NVP seems to be the main factor responsible for the increase of PON-1 activities induced by NVP exposure. This effect was only observed in the 3D model, suggesting that an *in vivo*-like system is necessary for this modulation to occur. The present data suggest that the 3D model is a more suitable *in vitro* model than the conventional ones to explore drug effects on PON-1.

2.2. Objectives

The objectives of the work described in the current Chapter were to explore the effects of NVP and its major phase I metabolites on PON-1 activities in three *in vitro* models of hepatocytes, and evaluate the contribution of biotransformation to PON-1 modulation induced by NVP.

2.3. Material and methods

2.3.1. Chemicals and reagents

NVP was purchased from *Cipla* (Mumbai, India). The NVP metabolites, 12-OH-NVP and 2-OH-NVP, were synthesized as described (Antunes et al., 2008; 2011). All other reagents were purchased from *Sigma-Aldrich* (Madrid, Spain) and used as received, unless stated otherwise.

2.3.2. Animal welfare and ethical statements

Female Wistar rats obtained from NOVA Medical School were used for the isolation of hepatocytes. All experiments involving rats were performed in agreement with the Basel Declaration, and according to the European Commission legislation on the protection and welfare of animals used for scientific purposes (Directive 2010/63/EU). All procedures involving animals received prior approval by the Ethics Committee of NOVA Medical School (reference number: 05/2013/CEFCM) and the National Authority (Direcção Geral de Alimentação e Veterinária, reference number: 0421/000/000/2013). The removal of hepatic tissue was conducted as humanely as possible, with the animals kept under anaesthesia.

2.3.3. 2D and 3D primary cultures of rat hepatocytes

Female Wistar rats, three to six months old (200–400 g), were kept in individual cages for at least 24 h prior to each experiment, with *ad libitum* access to food and water. Rat hepatocytes were isolated using a two-step perfusion-based collagenase method as previously described (Miranda et al., 2009). Briefly, the rats were anaesthetized with an intraperitoneal injection of an aqueous ketamine (90 mg/Kg body weight) and xylazine (10 mg/Kg body weight) solution (100 µL/Kg body weight). The liver was perfused via the vena portae for 10 min at 39 ˚C with perfusion buffer I (140 mM sodium chloride, 6.7 mM potassium chloride, and 10 mM HEPES), adjusted to pH 7.5 with 2.4 M ethylene glycol tetraacetic acid. Subsequently, perfusion was continued at 39 ˚C for 7 min with a

collagenase buffer, consisting of 0.2 mg/mL collagenase P in 67 mM sodium chloride, 6.7 mM potassium chloride, 100 mM HEPES, albumin (0.5% m/v) and 4.8 mM $CaCl_2 \cdot 2H_2O$ solution, adjusted to pH 7.6. The flow rate of the perfusion buffers was 10 mL/min. After perfusion, the liver was removed and dissociated in cold perfusion buffer I with 10 g/L of albumin. For enrichment of the final hepatocyte population, an additional Percoll® step was included by layering 5 mL of cell suspension over a 25% Percoll® (*GE Healthcare*, Little Chalfont, UK) solution. After centrifuging at 1300 *g* and 4 ˚C for 20 min, hepatocytes were obtained as a pellet. The Percoll® solution was removed by washing with PBS and the viability of the isolated hepatocytes was assessed by trypan blue exclusion; values within an 85–95% range were routinely obtained. To prepare 2D hepatocyte cultures 300000 cells/well were inoculated onto rat tail collagen-precoated 24-well culture plates in 0.5 mL of William's E medium supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 1% (v/v) NEAAs 100 × solution, 40 µg/mL gentamicin, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL amphotericin, 1.4 µM hydrocortisone, 15 mM HEPES, 0.3 mg/mL glutamine and 32 U/mL human insulin, designated as complete culture medium. Cell cultures were kept at 37 ˚C in a humidified atmosphere of 5% CO_2 in air. The culture medium was renewed every 24 h, and the cells were routinely examined under phase contrast microscopy before each culture medium renewal. The supernatants and cells were collected at day 4 and stored at −80 ˚C until subsequent assays. 3D hepatocyte spheroid cultures were performed in 125 mL spinner vessels as previously optimized (Miranda et al., 2009). Briefly, cells were cultured in complete culture medium at a cell density of 120000 cells/mL. Spinner vessels were maintained at 80 rpm on a magnetic stirrer, and kept at 37 ˚C in a humidified atmosphere of 5% CO_2 in air. The supernatants and cells were collected and stored at −80 ˚C until subsequent assays.

2.3.4. HepG2 cell cultures

HepG2 cells (ATCC® HB-8065™) were cultured in monolayer (2D) in 5 mL of alpha-minimum essential medium supplemented with 10% (v/v) FBS, 1% (v/v) NEAAs 100× solution and 1 mM sodium pyruvate, inoculated at a density of 20000 cells/cm^2 in 25

cm^2 culture flasks. HepG2 cell cultures were kept at 37 ˚C in a humidified atmosphere of 5% CO_2 in air. The supernatants and cells were collected at day 4 and stored at −80 ˚C for subsequent assays.

2.3.5. Incubation with nevirapine and phase I nevirapine metabolites

2D (HepG2 cells and rat hepatocytes) and 3D rat hepatocyte cultures were exposed to 300 µM NVP, 2-OH-NVP and 12-OH-NVP, for 4 days at 37 ˚C in a humidified atmosphere of 5% CO_2 in air. The compounds were administered in DMSO solution and the final DMSO concentration in cultures was 0.33% (v/v). The NVP concentration used in the experiments was based on the recommend oral dose of 400 mg once-daily for adult HIV-infected patients, divided by the volume of the vascular compartment of a 70 Kg adult (approximately 5 L). The concentration (300 µM) of each test compound (NVP, 2-OH-NVP and 12-OH-NVP) used in the experiments was sub-lethal, based on death curves of both HepG2 and primary hepatocytes (data not shown) and in accordance with other similar experiments performed with NVP (Mangiacasale et al., 2003; Pittoggi et al., 2008; Thein et al., 2014). The culture media from 2D primary rat hepatocyte cultures were totally replaced daily, while there was no medium replacement in 3D cultures during the 4-day experiment. HepG2 cells were also exposed to NVP, 2-OH-NVP and 12-OH-NVP to a final concentration of 300 µM. NVP and its metabolites were dissolved in DMSO, as described above. The cells were exposed to each compound for 4 days at 37 ˚C, in a humidified atmosphere of 5% CO_2 in air. In parallel, all cultures were incubated for 4 days with 0.33% DMSO that served as control.

2.3.6. Cytochrome P450 activity measurement

The EROD assay was performed in 2D and 3D rat hepatocyte cultures exposed to NVP, 2-OH-NVP and 12-OH-NVP (300 µM) for 4 days, as previously described (Donato et al., 1993), in order to measure the activity of CYP1A1/1A2 isozymes (Wilkening et al., 2003) as an indicator of metabolic competence of the different cell culture systems. Briefly, the procedure consisted on cell incubations with 8 mM 7-ethoxyresorufin in culture

medium for 90 min. The concentration of the product, 7-hydroxyresorufin, was measured in the supernatants after 2 h of enzymatic digestion with β-glucuronidase and arylsulfatase. The quantification was performed on the basis of fluorescence measurements (λexc: 530 nm; λem: 590 nm), using a calibration curve of resorufin in culture medium.

2.3.7. Medium collection and diafiltration

At day 4 of incubation with the test compounds, the cell culture supernatant was collected and centrifuged at 200 *g* for 5 min at 4 ˚C to remove cellular debris. In order to remove phenol red, which might interfere with the determination of PON-1 activities, diafiltration of the total cell culture supernatants against PBS was performed using Amicon® Ultra-4 centrifugal filter 10000 MWCO tubes (*Merck Millipore*, Billerica, USA) at 2000 *g* and 4 ˚C. After diafiltration, the supernatants of cell cultures were concentrated five-fold using the above mentioned 10000 MWCO devices and the concentrated (upper) fraction was used for PON-1 activity assays.

2.3.8. Paraoxonase-1 paraoxonase activity

The POase activity was assessed through quantification of *p*-nitrophenol formation from paraoxon, as previously described by Batuca and collaborators (2007). Briefly, freshly prepared paraoxon (1.0 mM) in 290 µL of 50 mM glycine buffer containing 1 mM $CaCl_2$ (pH 10.5) was incubated with 10 µL of sample, at 37 ˚C, for 10 min, in 96 well plates. *p*-Nitrophenol formation was monitored at 412 nm on a microplate reader (Biotrack II plate reader, *Amersham Biosciences*). The POase activity ($mU \cdot mL^{-1}$) was normalised by intracellular protein level.

2.3.9. Paraoxonase-1 arylesterase activity

The AREase activity was assessed by monitoring the extent of phenyl acetate hydrolysis into phenol and acetic acid (Figure 9), using a spectrophotometric method as previously

described by Dias and collaborators (2014b). Briefly, 10 µL of cell culture medium samples, or 10 µL of the standards, were diluted in the proportion of 1:2 in NaCl 0.9%. After 10 min of incubation at 37 ˚C, freshly prepared HEPES buffer (190 µL, 2 mM, pH 8), containing $CaCl_2$ (1 mM), BSA (0.005%), phenol red (106 µM) and phenyl acetate (5 mM), was added to each well. The absorbance was monitored at 405 nm. The AREase activity was obtained from the calibration curve and normalised using intracellular protein levels.

2.3.10. Paraoxonase-1 lactonase activity

The LACase activity was assessed by measuring the production of 3-(*o*-hydroxyphenyl) propionic acid from hydrolysis of the PON-1 substrate, dihydrocoumarin (Figure 9), using a spectrophotometric assay adapted to a 96-well microplate, as described by Dias and collaborators (2014a). Briefly, 10 µL of cell culture medium samples, or 10 µL of the standards, were diluted in the proportion of 1:2 in NaCl 0.9% and incubated for 10 min, at 37 ˚C. Then, freshly prepared HEPES buffer (190 µL, 2.0 mM, at pH 8.0) containing $CaCl_2$ (1.0 mM), BSA (0.005%), phenol red (106 µM) and dihydrocoumarin (1.0 mM) was added to each well. After 1 min of incubation at room temperature, the absorbance was measured at 405 nm. The activity was obtained from the calibration curve and normalised using intracellular protein levels.

2.3.11. Protein quantification

Culture biomass within 2D and 3D cultures was evaluated by quantification of total protein, as previously described (Santos et al., 2015). Briefly, after lysis of the cell pellet with 100 mM NaOH at 37 ˚C for 24 h, total protein was quantified using the BCA protein assay kit (*Novagen*, San Diego, CA, USA) according to manufacturer's specifications, by measuring absorbance at 280 nm (Micro-Drop LVis Plate, *BMG Labtech*, Germany) and using a suitable calibration curve. The total protein quantification was used to normalise the enzyme activities.

2.3.12. Data analysis and statistical procedures

Statistical analyses were performed using GraphPad®Prism version 5.0 (*GraphPad Software Inc*., La Jolla, CA, USA). The data were normalised by the amount of total protein in each sample. EROD activity was expressed relatively to control (DMSO 0.33%). The results are presented as the mean ± SEM of 5 replicates. The data were analysed by two-way ANOVA, except for comparisons of basal activities between the different model systems, which were performed by one-way ANOVA; a p value< 0.05 was considered significant.

2.4. Results

2.4.1. Ethoxyresorufin-*O*-deethylase activity was induced in the 3D culture model

Hepatic drug biotransformation is mostly the result of phase I and II enzymatic reactions. EROD activity assays that measure primarily CYP1A1 and 1A2 activities (Chang and Waxman, 2005) and to some extent, CYP2B activity (Jeong et al., 2004) were used to evaluate the metabolic competence of the 2D and 3D primary hepatocyte cultures and to disclose the influence, if any, of NVP, 2-OH-NVP and 12-OH-NVP on phase I enzymatic activity modulation in these systems. EROD activity (Figure 14) was detected in all instances and was induced in the 3D, but not in the 2D, cultures in the presence of all test compounds. The extent of EROD induction by NVP was 1.4-fold ($p< 0.05$, two-way ANOVA) and *ca.* 1.6-fold by both 2- and 12-OH-NVP ($p< 0.05$, two-way ANOVA), when compared to the control.

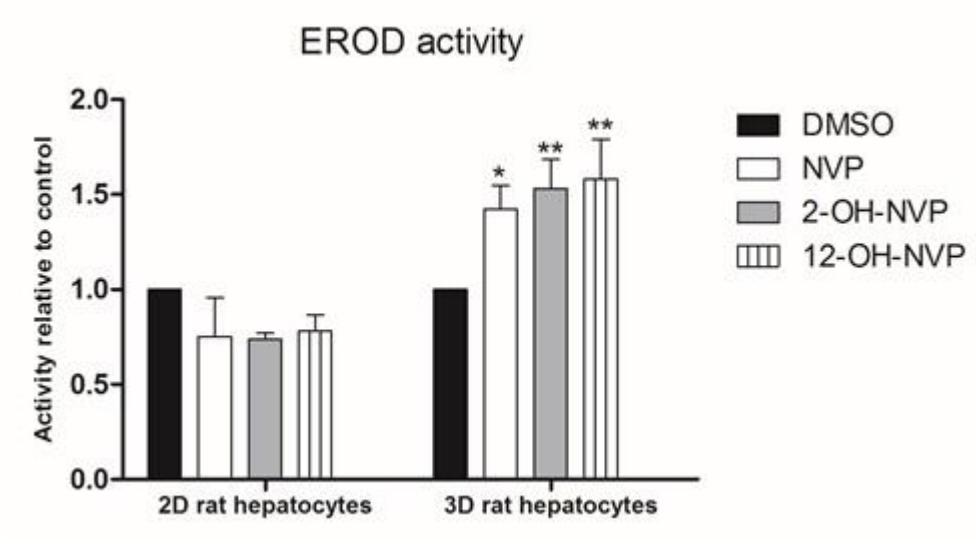

Figure 14 Ethoxyresorufin-O-deethylase activity expressed relatively to control in 2D and 3D rat hepatocyte cultures after treatment with nevirapine and its metabolites. *Ethoxyresorufin-O-deethylase (EROD) activity expressed relatively to the control [0.33% (v/v) dimethyl sulfoxide, DMSO] in 2D and 3D rat hepatocyte primary cultures after 4 days of treatment with nevirapine (NVP), 2-hydroxy-nevirapine (2-OH-NVP) and 12-hydroxy-nevirapine (12-OH-NVP) (n=5; *p< 0.05, **p< 0.01, two-way ANOVA).*

2.4.2. Paraoxonase-1 activities were modulated by nevirapine and 12-hydroxy-nevirapine in the 3D spheroid model

In this study PON-1 activities (POase, AREase and LACase) were assessed in both 2D and 3D rat hepatocyte cultures, as well as in HepG2 monolayer cell cultures exposed to NVP, 2-OH-NVP and 12-OH-NVP at 300 µM (Figure 15). The analyses were performed at day 4 of culture, when no decrease in cell viability was observed in any of the treated cultures (data not shown). The constitutive POase and AREase activities were found to be similar among groups, whereas the basal LACase activity was higher in the 2D model compared to the HepG2 system (p= 0.033, one-way ANOVA). Interestingly, as shown in Figure 15 Panel A, an increased POase activity compared to the control (two-way ANOVA) was only observed in 3D rat hepatocyte cultures treated with NVP (0.056 ± 0.012 mU/mg protein; p< 0.05) and 12-OH-NVP (0.074 ± 0.012 mU/mg protein; p< 0.01). Treatment with 2-OH-NVP had no effect, and neither the HepG2 nor the 2D rat hepatocyte model systems displayed POase activities above background upon exposure to any of the test compounds. Likewise, NVP and its metabolites had no significant effect on LACase activity in the 2D hepatocyte model and the HepG2 cells, while a significant increase was observed in the 3D spheroid model (Figure 15, Panel B, two-way ANOVA). Herein, a higher LACase activity was observed both in the presence of NVP (0.049 ± 0.010 U/mg; p< 0.05) and 12-OH-NVP (0.066 ± 0.003 U/mg; p< 0.001) but, again, 2-OH-NVP had no effect. Concerning the AREase activity (Figure 15, Panel C), both 2-OH-NVP and 12-OH-NVP led to an increasing activity trend in the 3D hepatocyte model when compared to the control. However, such difference was only statistically significant for 12-OH-NVP (0.118 ± 0.012 U/mg; p< 0.01). On the contrary, no alterations in PON-1 AREase activity were observed in HepG2 cells or in 2D hepatocyte primary cultures upon treatment with either NVP, 2-OH-NVP or 12-OH-NVP.

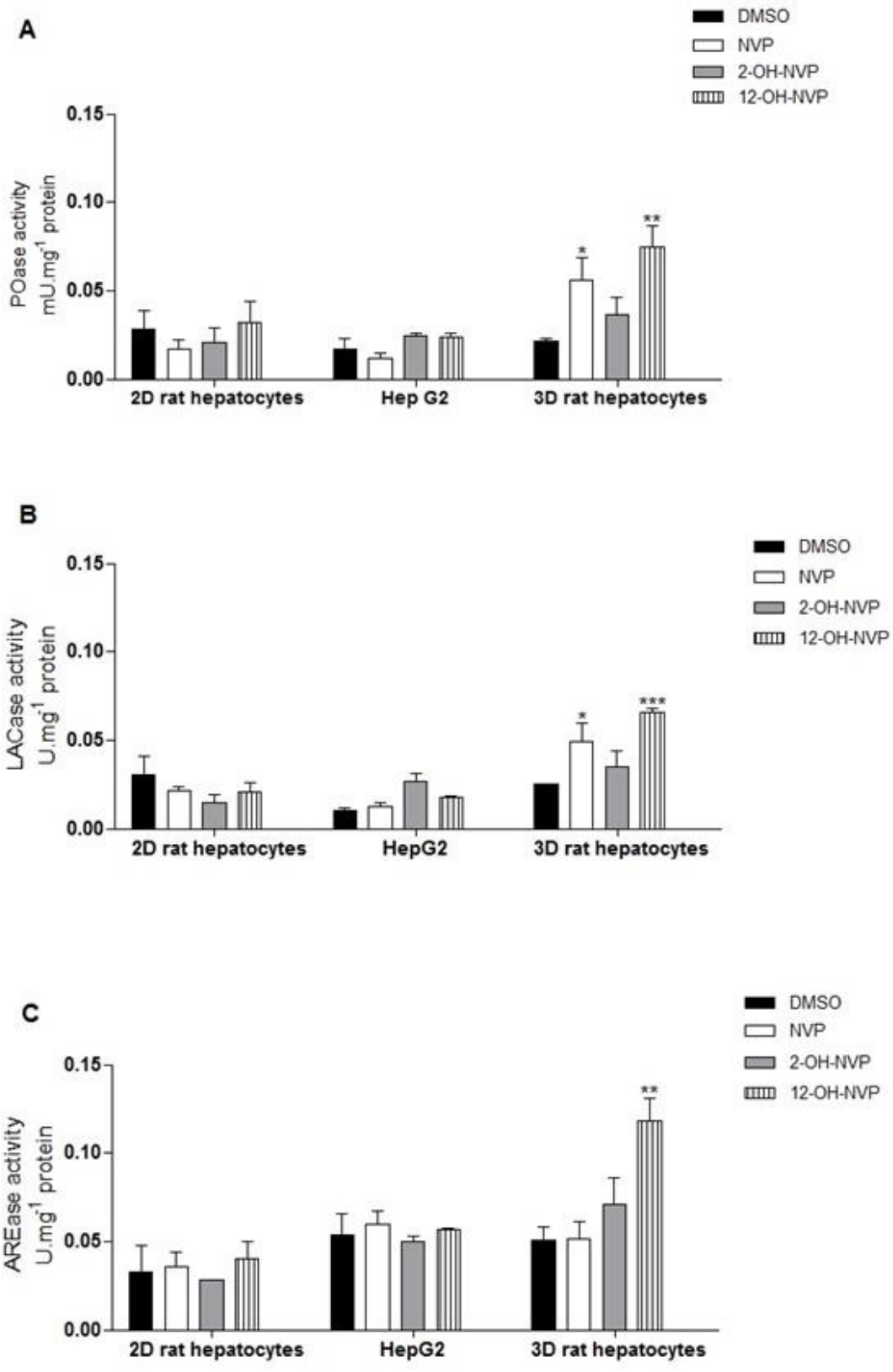

Figure 15 Paraoxonase-1 activities in HepG2 cells, 2D and 3D rat hepatocyte cultures after incubation with nevirapine and its metabolites. *Paraoxonase-1 (PON-1) activities assessed in HepG2 cells, 2D and 3D rat hepatocyte cultures after 4 days of incubation with nevirapine (NVP), 2-hydroxy-nevirapine (2-OH-NVP), 12-hydroxy-nevirapine (12-OH-NVP) (300 µM), and dimethyl sulfoxide (DMSO, 0.33% v/v, control). (A) Paraoxonase (POase, mU/mg protein), (B) lactonase (LACase, U/mg protein) and (C) arylesterase (AREase, U/mg protein) activities. Data are presented as the mean ± SEM (n=5). Two-way ANOVA, *p< 0.05, **p< 0.01, ***p< 0.001, was used for comparisons between test compounds and control.*

2.5. Discussion

In the present study, we investigated the effect of NVP and its phase I metabolites, 12-OH-NVP and 2-OH-NVP, on PON-1 POase, LACase and AREase activities using three different model systems, specifically HepG2 monolayer cell cultures, 2D and 3D rat hepatocyte cultures. We demonstrate herein that NVP and 12-OH-NVP increased POase and LACase activities, while 12-OH-NVP increased AREase activity when tested in the 3D rat hepatocyte model (Figure 16). It is important to note that, in all the three *in vitro* models, hepatocytes were exposed to a NVP concentration higher than the plasma concentrations observed in patients taking the drug. In fact, the steady-state plasma concentrations of NVP in these patients vary from 4 µM to 96 µM (Dickinson et al., 2014). However, the liver first-pass effect of this orally taken antiretroviral must be also taken into consideration. The liver tissue is exposed *in vivo* to a concentration of the drug much higher than the one measured in blood and available for distribution.

Although, constitutive POase, LACase and AREase activities were detected in the three *in vitro* models tested, the modulation of the different activities by NVP and 12-OH-NVP was only observed in the 3D hepatocyte model. Moreover, the formation of 12-OH-NVP seems to be the main factor responsible for the increase of PON-1 activities induced by NVP exposure. To the best of our knowledge, the current work is the first demonstration of an effect of NVP and its phase I metabolite, 12-OH-NVP, on PON-1 activities.

We were able to quantify the three PON-1 activities without drug exposure in all the *in vitro* models tested. The results indicated comparable levels for each activity in the three models (Figure 15), showing that PON-1 is constitutively secreted to a similar extent among models. The different enzymatic activities of PON-1 reflect its promiscuous nature and multiple physiological roles. The enzyme is involved in the metabolism of several xeno- and endobiotics. POase, the first activity to be discovered, is involved in the detoxification of environmental toxicants such as organophosphate derivatives and chemical warfare nerve agents (Valiyaveettil et al., 2012; Bigley and Raushel, 2013; Karlsson et al., 2015). Moreover, it has been considered as a marker of liver function (Camps et al., 2009; Hafez et al., 2014). The AREase activity is responsible for detoxifying lipoprotein-associated lipid peroxides (Aviram et al., 2000). Notably, the oxidised LDL has been indicated as a factor in early events of liver injury (Hammad et al., 2011). In

addition, the LACase activity is involved in protection from the deleterious effects of the toxic homocysteine metabolite, homocysteine-thiolactone, hence avoiding its accumulation and preventing homocysteine-thiolactone-induced apoptosis, endoplasmic reticulum stress and inflammation (Jakubowski, 1999). Homocysteine has also been reported as a biomarker of hepatotoxicity (Kubota et al., 2014). Furthermore, LACase activity has several other endogenous lactones substrates, such as 5,6-dihydroxyeicosatrienoic acid-1,5 lactone (a vasodilator), 5-hydroxy-eicosatetraenoic acid lactone (5-HETEL) (an inhibitor of phospholipase A2 and cyclooxygenase), 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid δ-lactone (an inhibitor of signal transduction pathways), or *N*-acyl homoserine lactones (involved in quorum sensing mechanisms) (Draganov and Teiber, 2008; Manolescu, 2013). For instance, 5-HETEL sensitises hepatocytes to tumour necrosis factor α-induced apoptosis and potentiates the apoptotic effects of drugs like actinomycin D (Martínez-Clemente et al., 2010).

Described as superior to the classic models, 3D hepatocyte cultures, that mimic *in vivo* tissue and maintain metabolic functions, are a good alternative to conventional *in vivo* pharmacological and toxicological studies. We have previously confirmed the metabolic competence of this model for NVP biotransformation studies. In fact, contrary to 2D cultures in which this was not observed, rat hepatocyte 3D cultures exposed to NVP (300 μM) yielded all known phase I NVP metabolites, at day 4, in the same relative proportions found in humans (Pinheiro et al., 2017). HepG2 cell lines, on the other hand, have been described as presenting important drawbacks regarding biotransformation ability and enzyme induction capacity. Accordingly, PON-1 modulation could only be observed in the 3D liver model in the present study, further emphasising that the selection of a relevant and competent *in vitro* model for mechanistic studies is crucial.

Higher POase and LACase activities were detected as a consequence of exposure to NVP and 12-OH-NVP, but not 2-OH-NVP, whereas AREase activity was increased only in 12-OH-NVP-treated cultures. A trend to higher activities upon exposure to 12-OH-NVP when compared to NVP-treated cultures was also observed, suggesting that 12-OH-NVP may have a role on PON-1 activity modulation, possibly as a result of phase II conjugation. These results are consistent with our previous demonstration that NVP biotransformation into 12-OH-NVP, as major metabolite, is quite efficient in the 3D rat hepatocyte model used herein (Marinho et al., 2014b).

PON-1 modulation by various drugs has been explored using different *in vitro* models and several mechanisms have been proposed to explain the observed effects. In particular, the activation of different transcription factors may underlie the induction of the PON-1 gene. For instance, PON-1 modulation by pitavastatin in a 2D culture of Huh7 cells involved the sterol regulatory element-binding protein-2 and the Sp1 transcription factor (Arii et al., 2009). In addition, acetylsalicylic acid, which was able to increase PON-1 expression and AREase activity in a 2D model of the HepG2 cell line and in primary rat hepatocytes, promotes PON-1 upregulation probably through the AhR (Jaichander et al., 2008). Similarly, an AhR-dependent mechanism was also described for quercetin- (Gouedard et al., 2004a) and resveratrol- (Gouedard et al., 2004b) induced modulation of PON-1 in Huh7 cell line cultures. Other pathways also proposed to be implicated in PON-1 modulation are the activation of the c-Jun transcription factor (Cheng et al., 2011), promoted by berberine in HepG2 cells, and the activation of the PPAR γ (Khateeb et al., 2010), caused by pomegranate polyphenols in the Huh7 cell line. Among the several pathways described above, the effect of NVP was only explored in PPAR γ expressed in human adipocyte precursor cells (Díaz-Delfín et al., 2011). NVP may promote the upregulation of PON-1 through a PPAR γ-dependent mechanism. Additionally, since NVP induces the CYP1A family and PON-1, involvement of the AhR receptor is also a possibility, as it is a target for induction by both enzymes (Guyot et al., 2012). In fact, the EROD activity assay that reflects primarily the activity of the CYP1A family (Figure 14) showed enzyme induction upon exposure to NVP and its metabolites; notably, this effect was only observed in the 3D model. Nevertheless, further studies should be conducted to elucidate the exact pathways involved in NVP-induced PON-1 modulation.

Herein, by resorting to a 3D rat hepatocyte model, we were able to show that NVP and its main phase I metabolite, 12-OH-NVP, have the ability to modulate PON-1 activities (Figure 16). However, these modulatory effects were observed using concentrations higher than the plasma concentrations observed in HIV-infected patients. To the best of our knowledge this is the first time that the usefulness of the 3D hepatocyte model to assess the modulation of PON-1 activities by drugs is described. This system will allow to further explore the mechanisms underlying these effects, paving the way for other research opportunities. For instance, PON-1 has been implicated in the pathogenesis of

several diseases (Table 3) and its modulation has been suggested for therapeutic purposes. Also, this model should allow the study of drug-drug interactions, drug-endogenous metabolites interactions and a better assessment of PON-1 as a marker of hepatic function.

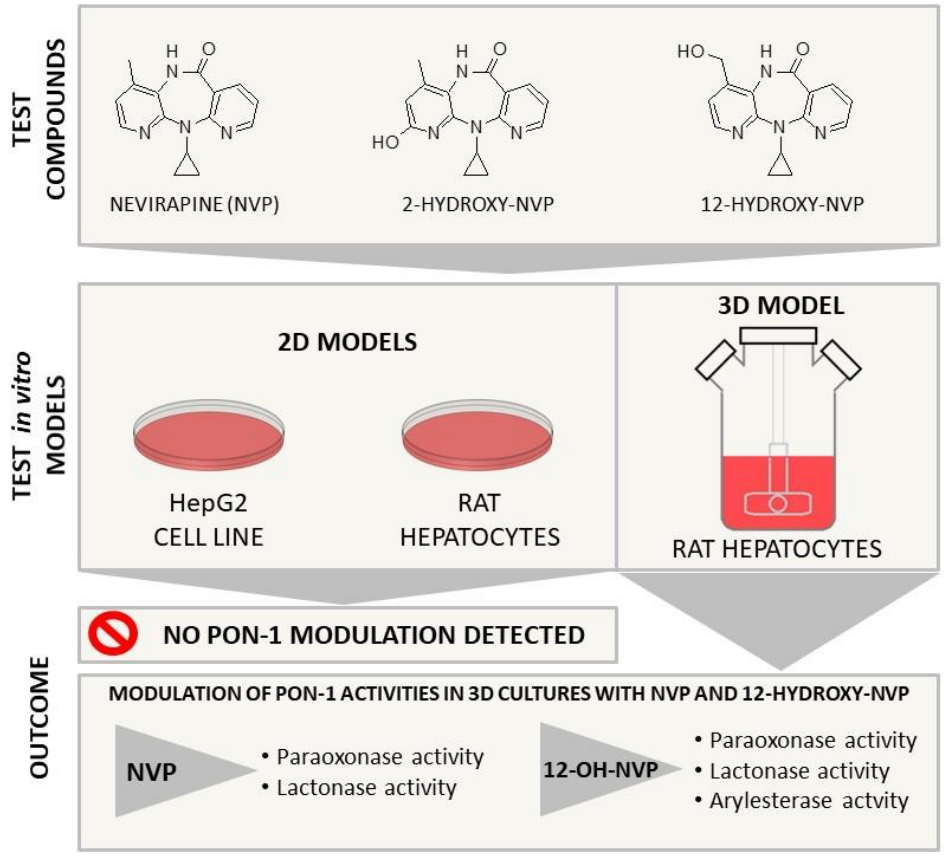

Figure 16 Effect of nevirapine and its metabolites in paraoxonase-1 activities in vitro. *Nevirapine (NVP) and its main phase I metabolites, 2-hydroxy-nevirapine (2-OH-NVP) and 12-hydroxy-nevirapine (12-OH-NVP), were incubated in three different in vitro models of hepatocytes: 2D cultures of the HepG2 cell line, 2D cultures of rat hepatocytes and 3D cultures of rat hepatocytes. The modulation of paraoxonase-1 (PON-1) activities was only possible in the metabolic competent 3D model. NVP was able to increase PON-1 paraoxonase (POase) and lactonase (LACase) activities, while the 12-OH-NVP metabolite was able to increase PON-1 POase, LACase and arylesterase (AREase) activities. The formation of the 12-OH-NVP metabolite seems to be the main factor in the modulation of PON-1 activities induced by NVP. In: Marinho et al., 2016.*

CHAPTER 3. THE APOLIPOPROTEIN A1 BOOSTER EFFECTS OF NEVIRAPINE ARE DUE TO THE MODULATORY PROPERTIES OF ITS METABOLITES

Chapter 3

The apolipoprotein A1 booster effects of nevirapine are due to the modulatory properties of its metabolites

3.1. Summary

There has been a growing interest in drugs to boost HDL. The antiretroviral drug NVP has been associated with HDL-friendly properties due to increases in ApoA1, being this effect sex-dependent, as it is NVP biotransformation. We hypothesised that the effect on ApoA1 is due to one of NVP metabolites and not due to NVP itself. To clarify the relevance of NVP biotransformation on ApoA1 modulation, three *in vitro* models of hepatocytes were tested, to minimise the limitations inherent to each model. NVP and its main phase I metabolites, 2-OH-NVP and 12-OH-NVP, were incubated in two-dimensional (2D) and 3D primary cultures of rat hepatocytes and 2D cultures of HepG2 cells. In the 3D rat hepatocyte model, there was a significant increase of ApoA1 levels after 12 days of exposure: up to 43% for NVP and up to 86% for 2-OH-NVP incubation. The effects of NVP and 2-OH-NVP were only observed in the metabolically competent 3D model, suggesting that biotransformation is crucial for the ApoA1 boosting effect. The delayed onset effects of 2-OH-NVP and the absence of effect in 2D models suggest that the formation of a phase II metabolite is probably responsible for ApoA1 modulation.

3.2. Objectives

The general aim of this Chapter was to clarify the effects of NVP and its major phase I metabolites, 2-OH-NVP and 12-OH-NVP, on ApoA1 levels in three *in vitro* models of hepatocytes, and investigate the individual contribution of each NVP metabolite and the contribution of NVP biotransformation to the modulatory effect of this antiretroviral on ApoA1 levels.

3.3. Material and methods

3.3.1. Chemicals and reagents

NVP was purchased from *Cipla* (Mumbai, India). The NVP metabolites, 2-OH-NVP and 12-OH-NVP, were synthesized as described elsewhere (Antunes et al., 2008; 2011). All the other chemicals and reagents were obtained from *Sigma-Aldrich* (Madrid, Spain).

3.3.2. Animal welfare

All the procedures involving animals were performed in agreement with the Directive 2010/63/EU, which regulates the use of animals for scientific purposes in the European Union, and also in agreement with the Basel Declaration. All the experiments involving rats received prior approval by the competent National Authority (*Direcção Geral de Alimentação e Veterinária*, DGAV, reference number: 0421/000/000/2013) and by the Ethics Committee of NOVA Medical School (reference number: 05/2013/CEFCM).

Female Wistar rats obtained from the animal facility of NOVA Medical School were used for the isolation of primary hepatocytes. The removal of hepatic tissue was performed as humanely as possible with the rats kept under anaesthesia during all the process. The general anaesthesia was administered by intraperitoneal injection (100 µL/Kg body weight); the anaesthetics used were ketamine (90 mg/Kg body weight) and xylazine (10 mg/Kg body weight).

3.3.3. 2D and 3D primary cultures of rat hepatocytes

Female Wistar rats, three to six months old (200 – 400 g), were kept in individual cages for at least 24 hours prior to each experiment with unrestricted access to fresh water and food. Rat hepatocytes were isolated using a two-step perfusion–based collagenase method as previously described (Miranda et al., 2009). To prepare 2D rat hepatocyte primary cultures, 300000 hepatocytes/well were inoculated onto rat tail collagen-precoated 24-well culture plates in 0.5 mL of complete culture medium, which is composed of William's E medium supplemented with 0.3 mg/mL glutamine, 1 mM

sodium pyruvate, 15 mM HEPES, 1% (v/v) non-essential amino acids (NEAA) 100x solution, 1.4 µM hydrocortisone, 32 U/mL human insulin, 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 40 µg/mL gentamicin and 100 µg/mL amphotericin. 2D rat hepatocytes cultures were kept at 37 ˚C in a humidified atmosphere of 5% CO_2. The culture medium was renewed every 24 h, and the cells were routinely examined under phase contrast microscopy before each culture medium renewal. 3D primary rat hepatocyte spheroid cultures were performed in 125 mL spinner vessels as previously optimized (Miranda et al., 2009). Rat hepatocytes were cultured in complete culture medium at a cell density of 120000 cells/mL. Spinner vessels were kept at 80 rpm on a magnetic stirrer, at 37 ˚C in a humidified atmosphere of 5% CO_2. The cells and supernatants were collected and stored at -80 ˚C until subsequent assays.

3.3.4. HepG2 cell cultures

HepG2 cells (ATCC® HB-8065™) were cultured in monolayer in alpha-minimum essential medium supplemented with 1 mM sodium pyruvate, 1% (v/v) NEAA 100x solution and 10% (v/v) FBS, inoculated at a density of 20000 cells/cm^2 in 25 cm^2 culture flasks. HepG2 cell cultures were kept at 37 ˚C in a humidified atmosphere of 5% CO_2. The cells and supernatants were collected and stored at -80 ˚C.

3.3.5. Incubation with nevirapine and its phase I metabolites

To evaluate the individual contribution of each metabolite to ApoA1 levels, 2D cultures of rat primary hepatocytes and HepG2 cells were exposed to 300 µM NVP or 300 µM 2-OH-NVP or 12-OH-NVP at culture day's 2, 4 and 8, and 2, 3 and 4, respectively. Similarly, 3D primary cultures of rat hepatocytes were incubated with equimolar concentrations of each compound, at days 4, 8 and 12. NVP and its metabolites were incubated in dimethyl sulfoxide (DMSO) solution and the final DMSO concentration in cultures was 0.33 % (v/v). In parallel, all cultures were incubated with 0.33% DMSO, as control. The cell media from 2D rat hepatocyte cultures were totally replaced daily, while the cell

media in 3D cultures were replaced every 4 days. The NVP concentration used in these experiments was based on previous work of our research team (Marinho et al., 2016; Pinheiro et al., 2017).

3.3.6. Medium collection and diafiltration

The cell culture supernatants collected after each incubation period were centrifuged at 200 *g* for 5 minutes at 4 ˚C to remove cellular debris. Diafiltration of the total volume of supernatant against PBS was performed using Amicon® Ultra-4 centrifugal filter 10000 MWCO tubes (*Merck Millipore*, Billerica, USA) at 2000 *g* and 4 ˚C. After this step, the cell culture supernatants were concentrated five-fold using the above mentioned 10000 MWCO tubes and the concentrated fraction (upper fraction) was used for the quantification of ApoA1 levels and for the quantification of total protein. Total protein was quantified for each sample of 2D and 3D cultures by spectrophotometry at 280 nm, using a microplate reader with UV/vis spectrometer (*Micro-Drop LVis Plate BMG Labtech*, Germany) and was expressed in mg/mL.

3.3.7. Apolipoprotein A1 quantification

ApoA1 was quantified in the supernatant of 2D and 3D rat hepatocytes cultures using the ELISA kit for quantification of rat ApoA1 (*Sunred Biological Technology*, Shanghai, China) according to the manufacturer's instructions. Similarly, the quantification of human ApoA1 in the supernatant of HepG2 cells was performed using an ELISA kit for the quantification of the human protein (*Sunred Biological Technology*, Shanghai, China). The concentration of ApoA1 in each sample was determined using an appropriate standard curve and was expressed in mg/mL.

3.3.8. Statistical analysis

Statistical analysis was performed using GraphPad®Prism version 5.0 (*GraphPad Software Inc*., La Jolla, CA, USA). The results are presented as the mean ± SEM of 5

replicates. Data was analysed by two-way ANOVA, except for comparisons of basal levels for the different *in vitro* models, which were performed by one-way ANOVA; a p value< 0.05 was considered significant.

3.4. Results

3.4.1. ApoA1 levels were increased by exposure to nevirapine and 2-hydroxy-nevirapine in the 3D spheroid model

In the current work, ApoA1 levels were quantified in 2D and 3D rat hepatocyte cultures and also in HepG2 monolayer cell cultures. Hepatocytes were exposed to NVP, 2-OH-NVP and 12-OH-NVP at a concentration of 300 µM and the levels of ApoA1 were normalised by the total amount of protein in the extracellular media. The hepatocytes were exposed to the test compounds up to 4, 8 and 12 days for HepG2 cultures, the 2D rat hepatocyte model and 3D rat hepatocyte model, respectively. At these timepoints for each *in vitro* model, no decrease in cell viability was observed for any of the treated cultures (data not shown), whereas the basal ApoA1 levels did not change significantly for non-treated cultures during the course of the experiment.

As shown in Figure 17, there was a time-dependent increase in ApoA1 levels in the 3D rat hepatocyte cultures upon exposure to NVP and 2-OH-NVP. The effect of 2-OH-NVP in the ApoA1 levels was observed after 12 days of incubation (0.13 ± 0.01; p< 0.001), when the ApoA1 levels in the cultures exposed to this metabolite were 86% higher than the ApoA1 levels in control cultures. Moreover, in comparison with non-treated hepatocytes, there was also an effect on ApoA1 levels after incubation with NVP. This increase in ApoA1 was more modest than upon 2-OH-NVP exposure, reaching a maximum effect of 43% increase in ApoA1 relatively to control (0.10 ± 0.01; p< 0.05) after 12 days of exposure to NVP.

For the 3D spheroid model, no effect of 12-OH-NVP on ApoA1 was observed (Figure 17). Neither NVP nor its metabolites affect ApoA1 levels in the 2D rat hepatocytes culture (Figure 18) or in the HepG2 cell line culture (Figure 19). The modulation of ApoA1 levels upon exposure to NVP and 2-OH-NVP was only possible in the 3D model, as no

modulatory effects of these compounds were noted in the 2D rat hepatocytes culture or in the HepG2 cultured in monolayer (Figures 18 and 19).

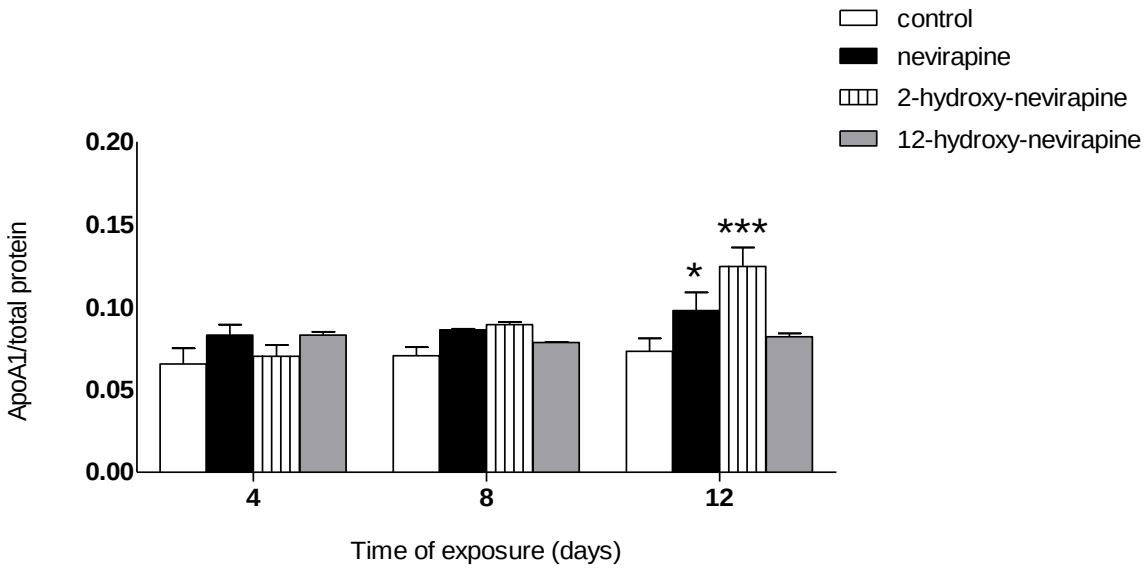

Figure 17*. *Apolipoprotein A1 quantification in the supernatant of 3D rat hepatocytes model at days 4, 8 and 12 of culture. *Hepatocytes in the 3D model were exposed to dimethyl sulfoxide (0.33%, control), nevirapine, 2-hydroxy-nevirapine, 12-hydroxy-nevirapine (300 µM) for up to 12 days. The statistical test for comparisons of apolipoprotein A1 (ApoA1) levels between control and test compounds was two-way ANOVA. Data is presented as mean ± SEM of ApoA1 levels normalised by protein in extracellular media. A p value < 0.05 was considered significant (* p < 0.05; *** p < 0.001).*

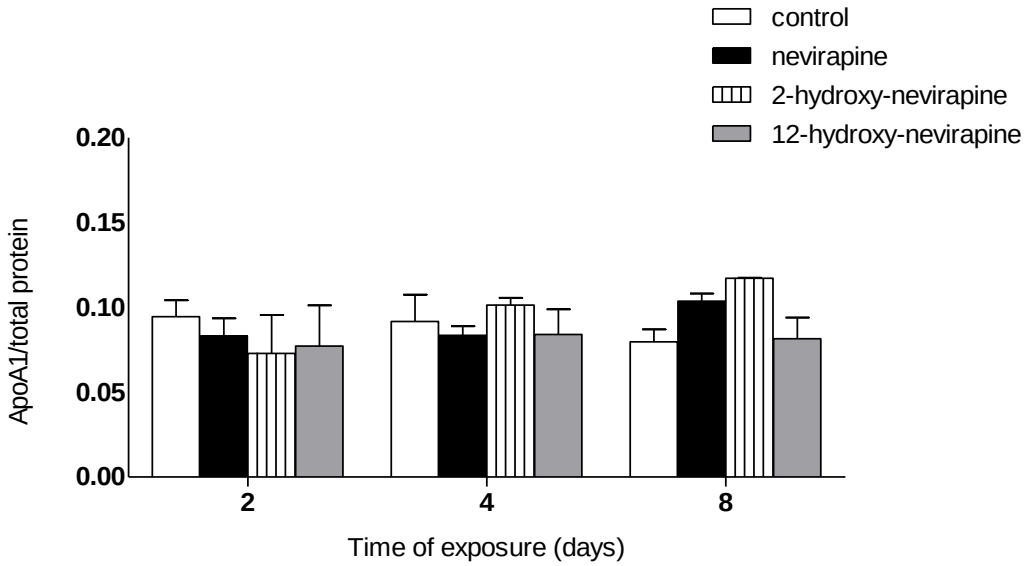

Figure 18. Apolipoprotein A1 quantification in the supernatant of 2D rat hepatocytes model at days 2, 4 and 8 of culture*. Hepatocytes were exposed to dimethyl sulfoxide (0.33%, control), nevirapine, 2-hydroxy-nevirapine, 12-hydroxy-nevirapine (300 µM) for up to 8 days. The statistical test for comparisons of apolipoprotein A1 (ApoA1) levels between control and test compounds was two-way ANOVA. Data is presented as mean ± SEM of ApoA1 levels normalised by protein in extracellular media.*

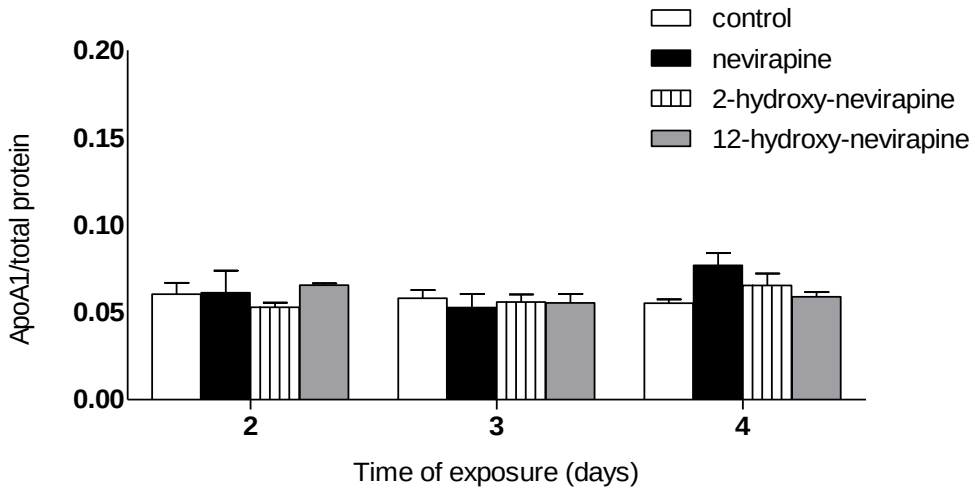

Figure 19. Apolipoprotein A1 quantification in the supernatant of 2D HepG2 cell line model at days 2, 3 and 4 of culture*. HepG2 cells were exposed to dimethyl sulfoxide (0.33%, control), nevirapine, 2-hydroxy-nevirapine, 12-hydroxy-nevirapine (300 µM) for up to 4 days. The statistical test for comparisons of apolipoprotein A1 (ApoA1) levels between control and test compounds was two-way ANOVA. Data is presented as mean ± SEM of ApoA1 levels normalised by protein in extracellular media.*

3.5. Discussion

In the current work, it is reported that the boosting effects of NVP on ApoA1 might be dependent on 2-OH-NVP formation and subsequent biotransformation. Moreover, this work shows the usefulness of a 3D hepatocyte model for the assessment of therapeutic targets modulation by drugs that undergo extensive biotransformation. To the best of our knowledge, this is the first time that a differential effect of NVP and its metabolites on ApoA1 levels is studied.

Our experimental approach was based on three different *in vitro* hepatocyte models, which were employed to investigate the effects of NVP and its main phase I metabolites, 2-OH-NVP and 12-OH-NVP on ApoA1 levels. It was demonstrated that NVP and particularly the 2-OH-NVP metabolite were able to increase the levels of ApoA1 on the hepatocytes supernatant, but only in the 3D model and after 12 days of incubation (Figure 20). Nonetheless, the modulation of ApoA1 by NVP and 2-OH-NVP was only observed in the 3D hepatocyte model, the basal levels of this apolipoprotein were quantified in all the *in vitro* models tested. In fact, the 3D hepatocyte cultures, unlike monolayer cultures, can mimic the *in vivo* cellular organization, the tissue physiology and the liver metabolic functions, being a suitable alternative model to *in vivo* pharmacological and toxicological research (Miranda et al., 2009). Furthermore, the metabolic competence of the 3D rat hepatocyte model for NVP biotransformation and bioactivation was previously confirmed by our research team (Pinheiro et al., 2017). In contrast, human cell lines such as HepG2, have important disadvantages for exploring the effects of xenobiotics that suffer extensive biotransformation and bioactivation due to the reduced metabolic ability of these cells (Gerets et al., 2012). The results presented here demonstrate that, only in metabolically competent hepatocyte models, NVP and 2-OH-NVP were able to modulate ApoA1 levels. Thus, the biotransformation of NVP and 2-OH-NVP seems to be crucial for this ApoA1 boosting effect.

NVP biotransformation involves extensive phase I and phase II reactions (Figure 7). Firstly, NVP undergoes oxidative metabolism via several CYP450 isozymes, generating five hydroxylated metabolites (Erickson et al., 1999; Riska et al., 1999a, 1999b). The main phase I metabolites of NVP, 2-OH-NVP and 12-OH-NVP, are generated mainly

through CYP3A4 metabolism (Figure 7; Riska et al., 1999a; 1999b). Recently our research team reported that in 3D cultures of hepatocytes following the incubation with NVP, the levels of free 2-OH-NVP and 12-OH-NVP remain stable from day 4 to day 12 of culture. However, when these 3D hepatocytes are incubated with 2-OH-NVP, therefore in the absence of NVP conversion into 2-OH-NVP or other phase I metabolites, the levels of free 2-OH-NVP decrease substantially until day 12 of culture (Pinheiro et al., 2017). This points towards a continuous phase I biotransformation of NVP into 2-OH-NVP in this 3D model, thus keeping the levels of phase I metabolites constant throughout the curse of the experiment, despite of phase II conjugation of phase I metabolites. These observations also suggest that 2-OH-NVP is an inducer of its phase II metabolism (Pinheiro et al., 2017). In contrast, when 2D rat hepatocytes are incubated with NVP, the levels of phase I metabolites are significantly lower than in 3D hepatocytes (Pinheiro et al., 2017), demonstrating the remarkable differences between these two models in terms of metabolic capacity and ability to promote the induction effects of NVP. Accordingly, it was observed CYP450 enzymes induction in 3D cultures upon exposure to NVP, as demonstrated by the ethoxycoumarin O-deethylase (ECOD) and ethoxyresorufin-O-deethylase (EROD) activities assays (Marinho et al., 2016; Pinheiro et al., 2017).

Subsequently, the phase I metabolites of NVP can undergo phase II biotransformation, namely glucuronoconjugation (Riska et al., 1999a, 1999b; Pinheiro et al., 2017) and sulfoconjugation (Chen et al., 2008; Sharma et al., 2013; Pinheiro et al., 2017). It was recently shown in 3D cultures of hepatocytes exposed to NVP and to 2-OH-NVP, a clear metabolic shift from glucuronoconjugation to sulfoconjugation throughout the course of the experiment (Pinheiro et al., 2017). Thus, at day 12 of culture, the concentration of 2-OH-NVP sulfate reaches its maximum levels, with only negligible levels of 2-OH-NVP glucuronide (Pinheiro et al., 2017). In accordance with these observations, in the 3D hepatocyte model exposed to NVP and to 2-OH-NVP, the activity of SULT1A1 is significantly induced from day 2 to day 10 of culture (Pinheiro et al., 2017). This induction of SULT1A1 with 2-OH-NVP sulfate formation was only observed in the 3D model of hepatocytes (Pinheiro et al., 2017). In the current study, in the 3D hepatocytes exposed to 2-OH-NVP, it was observed a gradual increase in ApoA1 levels over time, reaching its

maximum effect at day 12. This is in agreement with a gradual boosting of ApoA1 along with the 2-OH-NVP sulfate accumulation. Also, this is in concordance with the effect observed for cells exposed to NVP; in which higher ApoA1 levels were obtained at day 12, albeit with a lower magnitude than 2-OH-NVP. Therefore, the modulation of ApoA1 levels in hepatocytes possibly occurs upon NVP conversion into 2-OH-NVP, followed by SULT-mediated biotransformation of 2-OH-NVP and accumulation of a 2-OH-NVP sulfate. This modulatory effect on ApoA1 was only observed in the metabolic competent 3D hepatocyte model, although we cannot exclude higher accumulation of metabolites in the 3D model as compared to the others 2D cultures, due to differences in cell culture conditions, namely in the frequency of cell media changes.

In addition, there is *ex vivo* evidence showing that the intestinal contribution for NVP biotransformation is significant, particularly concerning the conversion of NVP into 2-OH-NVP by the enterocytes from female rats (Pinheiro et al., 2015). Consequently, increased levels of 2-OH-NVP can reach the liver in females. This increased availability of 2-OH-NVP, together with the increased ability for sulfoconjugation observed for the female sex (Tsoi et al., 2001; Wu et al., 2001; Alnouti and Klaassen, 2006; Hirao et al., 2011; Suzuki et al., 2012), might explain the observed sex-differences on the effect of NVP treatment on ApoA1 levels, being the ApoA1 booster effect of NVP higher in HIV-infected women (*cf.* Chapter 1; Marinho et al., 2014a). Moreover, it is reported significant SULT activity in the small intestine, including SULT1A1, 1A3, 1E1 and 2A1 (Chen et al., 2003), and there is also synthesis of lipid-free ApoA1 by the small intestine (Kingwell et al., 2014). Therefore, the contribution of intestinal biotransformation for the ApoA1 boosting effect observed in patients under NVP treatment should not be neglected. Furthermore, ApoA1 was shown to increase the stability of SULT2B1, at least in platelets (Yanai et al., 2004). Although it is not clear if this stabilising effect of ApoA1 is relevant for SULTs activity in other tissues, such as liver or small intestine, this could constitute a possible mechanism in ApoA1 induction by 2-OH-NVP, with ApoA1 contributing for the sulfoconjugation of 2-OH-NVP.

The positive effect of NVP-based cART on HIV-infected patients' lipid profile and HDL-cholesterol has been extensively described in literature (van der Valk et al., 2001; Clotet et al., 2003; van Leth et al., 2004; Sankatsing et al., 2007; Floridia et al., 2009; Franssen

et al., 2009; Podzamczer et al., 2011, 2012, 2014; Soriano et al., 2011; Strehlau et al., 2012; Arpadi et al., 2013). It is also described the beneficial impact of NVP-based treatment on HDL particle size (van der Valk et al., 2001; Clotet et al., 2003; Franssen et al., 2009). Some studies assessed ApoA1 levels in patients on NVP-based therapy (van der Valk et al., 2001; Clotet et al., 2003; Sankatsing et al., 2007; Franssen et al., 2009; Podzamczer et al., 2011; Soriano et al., 2011) however none of these works evaluated the underlying mechanism and the effect of different NVP metabolites on ApoA1. Although not considering the effect of NVP biotransformation, Franssen and co-authors, employing a methodology based on stable isotope-labelled tracers for kinetic analysis of HDL-ApoA1, have shown that the increase of HDL levels following NVP treatment is due to the stimulation of ApoA1 synthesis, with no changes on the catabolic rate of this apolipoprotein (Franssen et al., 2009).

Different intracellular pathways may be underlying this effect on ApoA1, and the activation of different transcription factors can promote the upregulation of this apolipoprotein. As previously mentioned, NVP is an inducer of its own metabolism (Lamson et al., 1999; Faucette et al., 2006; Pinheiro et al., 2017). It has been recognised that several inducers of CYP450, namely CYP3A4 inducers, such as phenobarbital, phenytoin or gemfibrozil, are able to increase ApoA1 production and HDL levels in a clinical setting (Luoma, 2008). In fact, the activation of certain transcription factors can lead to the upregulation of metabolising enzymes and ApoA1, as these genes are often targets of the same molecular pathways. For example, the activation of peroxisome proliferator-activated receptor γ (PPAR γ) can lead to the upregulation of hepatic ApoA1 (Dahabreh and Medh, 2012) and is possibly involved in NVP-associated induction of CYP3A4 and CYP2B6 (Rogue et al., 2010). Similarly, the activation of PPAR α leads to the upregulation of hepatic ApoA1 (Mukherjee et al., 2008) and CYP3A4 (Thomas et al., 2013). Additionally, PPAR α activation also induces the expression of hepatic SULT2A1 (Fang et al., 2005). However, if any NVP metabolite has PPAR γ or PPAR α agonist properties, remains unaddressed. In addition, ApoA1 expression can also be achieved through the aryl hydrocarbon receptor (AhR) (Jaichander et al., 2008). Since 2-OH-NVP is able to induce target genes of AhR, such as the CYP1A family, in 3D cultures of hepatocytes (*cf*. Chapter 2; Marinho et al., 2016; Pinheiro et al., 2017), the involvement

of this nuclear receptor on CYP450 and ApoA1 modulation should be considered. Besides the above mentioned nuclear receptors, CAR is also involved on CYP450 and ApoA1 modulation. In fact, it is described that NVP can promote the expression of CYP3A4 and CYP2B6 through CAR activation (Faucette et al., 2006). Moreover, it is reported that CAR activation can lead to increased hepatic expression of several SULTs, particularly in female mice (Alnouti and Klaassen, 2008). However, unlike the previously cited pathways, the activation of CAR causes decreased activity of the human ApoA1 promoter, and decreased hepatic expression and plasma levels of human ApoA1 in transgenic mice (Masson et al., 2008). At first glance, this observation may seem conflicting with the effect of NVP on ApoA1. However, the effect of NVP metabolites was never assessed in regard to this nuclear receptor. It is possible that a small molecule, which acts as an agonist for a given receptor, can lose agonistic activity upon small chemical modifications, as already described for other drugs (Spang et al., 2000). In addition to the previously mentioned pathways, ApoA1 expression can also be promoted through the LXR (Kannisto et al., 2014), pregnane X receptor (PXR) (Bachmann et al., 2004) and also by activation of the liver receptor homolog-1 (LRH-1) (Delerive et al., 2004). There is evidence that NVP does not activate LXR (Svärd et al., 2014) and is a weak activator of PXR (Faucette et al., 2006); however, once again, the effects of NVP metabolites on LXR and PXR have not been explored so far. Similarly, the activation of LRH-1 by NVP or its metabolites remains unexplored.

Besides the regulation of gene expression, another mechanism that can lead to increased ApoA1 levels is based on hepatocyte ApoA1 secretion. It was reported that the inhibition of the α-subunit of farnesyltransferase (FNT) promotes an increased secretion of ApoA1 by HepG2 cells and higher ApoA1 serum levels in transgenic mice expressing human ApoA1 (Miles et al., 2013). Interestingly, the PIs lopinavir and atazanavir, two anti-HIV drugs associated with CYP3A4 inhibition and pro-atherogenic changes on patients' lipid profile, are not able to inhibit FNT *in vitro* (Coffinier et al., 2007). Additional studies are needed to address if NVP and its metabolites have some degree of FNT inhibition ability.

ApoA1 is the main protein of HDL particles and a key component for their functionality (Nguyen et al., 2013; Kingwell et al., 2014). The pleiotropic effects of ApoA1 (Tuteja and Rader, 2014) substantiate the importance of this protein for several physiological

functions, far beyond cholesterol homeostasis; although the best known function of ApoA1 is on reverse cholesterol transport (Figure 2). The binding of ApoA1 to phospholipids and cholesterol, to generate pre-β HDL particles, is mediated by the hydrophobic C-terminal domain of ApoA1 (Nguyen et al., 2013). The lipid enrichment of these pre-β HDL particles might occur upon interaction between ApoA1 and the ABCA1 transporter, though an ABCA1-independent pathway is also possible, via ApoA1 interaction with the ABCG1 transporter or with SR-B1. In fact, it was reported that NVP intake is able to stimulate ABCA1-independent cholesterol efflux from macrophages (Tohyama et al., 2009). These interactions will promote the transfer of unesterified cholesterol and phospholipids from peripheral tissues to HDL, being ApoA1 a lipid acceptor in this process of cholesterol efflux (Vedhachalam et al., 2007; Kingwell et al., 2014; Mei and Atkinson, 2015). In addition, ApoA1, acting as a co-factor for LCAT, promotes the esterification of free cholesterol into the HDL particle, generating spheroidal HDL with a core of cholesteryl esters (Kunnen and Van Eck, 2012). It was reported that NVP treatment can promote a slight increase in LCAT activity in HIV-infected patients (Franssen et al., 2009).

Besides its fundamental role in cholesterol homeostasis, ApoA1 has also other essential physiological functions that have been investigated in the last years (Srinivas et al., 1991; Tada et al., 1993; Ariel et al., 1994; Huang et al., 2008; Bogan and Hennebold, 2010; Sriraman et al., 2010; Wilhelm et al., 2010; Karavia et al., 2012; Kim et al., 2016). Therefore, the modulation of ApoA1 levels might have several potential therapeutic applications. HDL- and ApoA1-targeted therapies were already suggested for myocardial infarction (Van Linthout et al., 2015), atherosclerosis (Millar and Cuchel, 2015; Gadkar et al., 2016) and more recently for cancer therapy (Zamanian-Daryoush and DiDonato, 2015). Even so, and despite the undeniable therapeutic potential of such ApoA1-targeted approaches (Gadkar et al., 2016), to date there are no drugs to effectively increase HDL or ApoA1 levels (Table 1). An example of the attempts at developing a new HDL/ApoA1 modulator is the drug RVX-208. The RVX-208 is a specific inhibitor of the second bromodomains within the BET proteins; by inhibiting the second bromodomains RVX-208 will promote the displacement of BET proteins from chromatin, theoretically leading to increased transcription of ApoA1 (Picaud et al., 2013). However, the ASSURE clinical trial showed no incremental effect of RVX-208 on ApoA1 or HDL levels, compared

with placebo, and no clinical improvement in terms of coronary atherosclerosis (Nicholls et al., 2016). Another example of a failed attempt to develop a drug with HDL-boosting properties is the use of CETP inhibitors. Torcetrapib and evacetrapib are CETP inhibitors, which block the transfer of cholesteryl esters from HDL to other lipoproteins through inhibition of CETP, then raising HDL-cholesterol levels. Both drugs were associated with significant increases in HDL-cholesterol, however torcetrapib was also associated with toxicity due to off-target effects, including increased risk of death, that was considered unacceptable (Barter et al., 2007); while evacetrapib was associated with hypertension and no clinical benefits (Nicholls et al., 2011a; Eyvazian and Frishman, 2017). Unlike these new molecules, the pathways that lead to NVP-induced toxicity are becoming better understood. Indeed, NVP-associated toxicity is dependent on its bioactivation into reactive electrophiles (Chen et al., 2008; Antunes et al., 2010a; Sharma et al., 2013), which are prone to form adducts with biomolecules (Antunes et al., 2008, 2010a, 2010b; Caixas et al., 2012; Meng et al., 2013; Sharma et al., 2013; Pinheiro et al., 2017) eliciting immune-mediated adverse reactions (Yuan et al., 2011). Moreover, as demonstrated in the current work, the effect of NVP on ApoA1 might be mediated via 2-OH-NVP. This metabolite can be bioactivated *in vitro* into potentially reactive metabolites (Antunes et al., 2011; Harjivan et al., 2015), although the *in vivo* relevance of this bioactivation pathway to the NVP toxic outcome remains to be elucidated. On the other hand, the effect of NVP on HDL and ApoA1 levels are well recognised and extensively described. Therefore, a 2-OH-NVP derivative might eventually lead, in the future, to the development of a new ApoA1-raising drug, with the beneficial features of NVP but with decreased potential for toxicity.

In conclusion, the modulatory effects of 2-OH-NVP were only observed in the 3D hepatocyte model after 12 days of incubation (Figure 20), suggesting that further biotransformation of 2-OH-NVP might be involved. The current study proves the usefulness of a system able to mimic the tissue physiology, for the study of the ApoA1 modulation by small molecules that can undergo significant biotransformation.

Considering the sex-dependent dimorphic profile of NVP-induced ApoA1 modulation (Chapter 1; Marinho et al., 2014a), we conjectured that NVP or an analogue might have a therapeutic application in pathologies affecting particularly women and characterised

by ApoA1 deficiency. In addition, NVP shows female-predominant toxicity (Marinho et al., 2014c) as well as anti-proliferative properties (Mangiacasale et al., 2003; Hecht et al., 2015). These features prompted us to investigate ovarian cancer, a women-specific malignancy with dismal prognosis characterised by decreased ApoA1 in serum and in the ovarian tissue (Wegdam et al., 2014). This work is reported in the following Chapter.

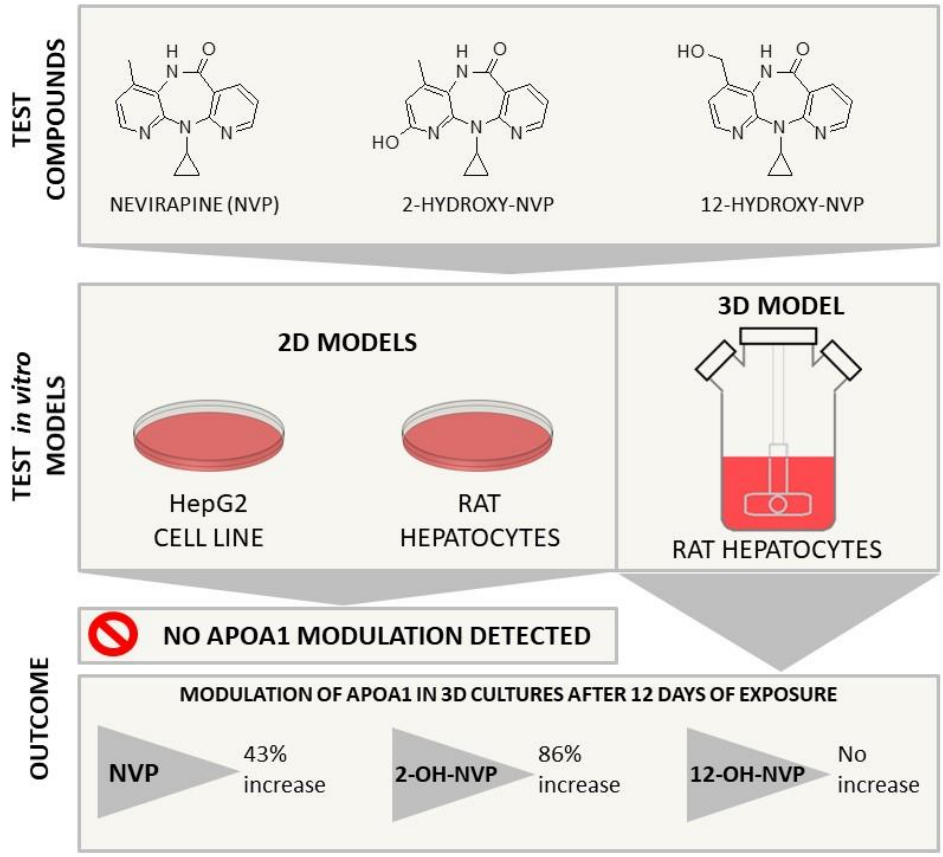

Figure 20 Effect of nevirapine and its metabolites in apolipoprotein A1 levels in vitro. *Nevirapine (NVP) and its main phase I metabolites, 2-hydroxy-nevirapine (2-OH-NVP) and 12-hydroxy-nevirapine (12-OH-NVP), were incubated in three different in vitro models of hepatocytes: 2D cultures of the HepG2 cell line, 2D cultures of rat hepatocytes and 3D cultures of rat hepatocytes. The modulation of hepatic apolipoprotein A1 (ApoA1) was only possible in the metabolic competent 3D model, after 12 days of incubation with NVP and 2-OH-NVP. NVP was able to increase ApoA1 levels by 43%, while the 2-OH-NVP metabolite was able to increase the levels of this protein by 86%. The NVP-induced increase in ApoA1 levels seems to be mediated via 2-OH-NVP. Adapted from Marinho et al., 2016.*

CHAPTER 4. ANTI-TUMORIGENIC EFFECTS OF APOLIPOPROTEIN A1 AND APOLIPOPROTEIN A1 MIMETIC PEPTIDES: PRELIMINARY RESULTS IN OVARIAN CANCER

Chapter 4

Anti-tumorigenic effects of apolipoprotein A1 and apolipoprotein A1 mimetic peptides: Preliminary results in ovarian cancer

4.1. Summary

ApoA1 is remarkably decreased in serum and ovarian tissues of women suffering from ovarian cancer; moreover, higher serum ApoA1 levels are associated with better prognosis and longer survival for ovarian cancer patients. In fact, ApoA1 and ApoA1 mimetic peptides are able to sequestrate pro-inflammatory phospholipids, some of which are known to activate a variety of signalling pathways involved in cancer cells proliferation, invasion, survival and chemoresistance. Besides, more intrinsic anti-tumorigenic properties, independent from ApoA1-lipids interaction, were also described for this protein. In the current study, our aim was to disclose the effects of ApoA1 and an ApoA1 mimetic peptide on the malignant phenotype of ovarian cancer cells, particularly regarding cell viability, invasion of extracellular matrix and ability to sensitise cells to platinum-based chemotherapy. Both ApoA1 and the mimetic peptide significantly decreased the viability of ovarian cancer cell lines. Importantly, ApoA1 was able to significantly decrease ovarian cancer cells invasiveness, while the ApoA1 mimetic peptide strongly decreased Akt phosphorylation at Ser^{473}, being able to sensitise ovarian cancer cells to cisplatin. The results reported in here support the role of ApoA1 and ApoA1 mimetic peptides as suppressors of ovarian tumorigenesis. Based on these preliminary findings further studies are needed to elucidate the usefulness of ApoA1 boosters for treating ovarian cancer, a gynaecological malignancy associated with dismal prognosis, for which the outcome of currently available therapies remains largely unsatisfactory.

4.2. Objectives

The research work described in the current Chapter was aimed at disclosing the effects of ApoA1 and an ApoA1 mimetic peptide (Ac-$F^{3,14}$18A-NH_2; also known as 4F) on the

malignant phenotype of ovarian cancer cells, focusing on cancer cells viability, invasiveness and also evaluating their potential to induce sensitisation of ovarian cancer cells to cisplatin.

4.3. Material and methods

4.3.1. Chemicals and reagents

ApoA1 extracted from human plasma, RPMI-1640 and DMEM media, RIPA buffer and bovine insulin were purchased from *Sigma-Aldrich* (MO, USA). The ApoA1 mimetic peptide (4F or Ac-$F^{3,14}$18A-NH_2; peptide sequence: acetyl-DWFKAFYDKVAEKFKEAF-NH_2 synthesized from all L aminoacids) was synthesized by *JPT Peptide Technologies* (Berlin, Germany). L-glutamine and Penicillin/Streptomycin solutions were purchased from *Invitrogen* (CA, USA). The Minisart® NML Syringe Filters with surfactant-free cellulose acetate membrane, pore size 0.2 µm, were supplied by *Sartorius* (Göttingen, Germany). The CellTiter 96® AQueous One Solution Cell Proliferation assay and the ApoTox-Glo™ triplex assay were provided by *Promega* (WI, USA). The Matrigel® Growth Factor Reduced Basement Membrane Matrix, Phenol Red-free, was supplied by *BD Biosciences* (NJ, USA). The protease inhibitor cocktail was obtained from *Roche* (Basel, Switzerland) and the phosphatase inhibitor cocktail II was obtained from *Calbiochem* (CA, USA). Pierce™ BCA Protein Assay Kit for protein quantification and BSA Microbiological Grade Powder were supplied by *Thermo Fisher Scientific* (MA, USA). The Immobilon® polyvinylidene difluoride (PVDF) membranes for western blot and the Immobilon™ Western Chemiluminescent HRP Substrate reagents were provided by *Millipore* (MA, USA). Phospho-Akt Ser^{473} (D9E) XP® rabbit monoclonal antibody, Akt (pan) (11E7) rabbit monoclonal antibody, Phospho-p44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}) (D13.14.4E) XP® rabbit monoclonal antibody and p44/42 MAPK (ERK1/2) (137F5) rabbit monoclonal antibody were purchased from *Cell Signaling Technology* (MA, USA). Calnexin antibody was purchased from *ENZO Life Sciences* (NY, USA). Dako Horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit IgG was supplied by *Agilent Technology* (CA, USA). Ovarian cancer cell lines were supplied by *ATCC* (VA, USA), while OSEC2 cell line was kindly provided by Dr. Richard Edmondson (Newcastle University, UK).

4.3.2. Cell lines and cell culture

SKOV3 cells (ATCC® HTB-77™) were maintained in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine and 50 U/mL penicillin/streptomycin. OVCAR3 cells (ATCC® HTB-161™) were maintained in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin and 0.01 mg/mL bovine insulin. CAOV3 cells (ATCC® HTB-75™) were maintained in DMEM media supplemented with 10% FBS, 2 mM L-glutamine and 50 U/mL penicillin/streptomycin. SKOV3, OVCAR3 and CAOV3 cells were cultured at 37 ˚C in a humidified atmosphere of 5% CO_2. Immortalized non-neoplastic human ovarian surface epithelial cells (OSEC2) were kept in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine and 50 U/mL penicillin/streptomycin, at 33 ˚C in a humidified atmosphere of 5% CO_2, as previously described (Davies et al., 2003; McKie et al., 2012). All cell lines were tested regularly to exclude mycoplasma contamination.

4.3.3. Preparation of stock solutions

Stock solutions of 1 mg/mL ApoA1 were prepared in 50% serum-free RPMI-1640 media in deionized distilled water. Stock solutions of 1 mg/mL ApoA1 mimetic peptide were prepared in 50% serum-free RPMI-1640 media in deionized distilled water or 50% serum-free DMEM media in deionized distilled water. After sterilizing filtration through a 0.2 µm filter, stock solutions were stored at -20 ˚C. These stock solutions were diluted in appropriate cell culture media to obtain the test concentrations.

4.3.4. Cell viability assay and caspase-dependent apoptosis assay

The effect of human ApoA1 on cell mitochondrial viability and caspase 3/7 activation was investigated in SKOV3 cells. Briefly, SKOV3 cells were seeded in 96-well plates, at a density of 5000 cells/well in full media conditions, and were allowed to attach for 24 h at 37 ˚C in a humidified atmosphere of 5% CO_2. Cell media were then changed to serum-free RPMI-1640. After overnight serum starvation, cells were incubated with increasing concentrations of ApoA1 (1 – 100 µg/mL) for 48 h. Mitochondrial viability was measured

by CellTiter 96® AQueous One Solution Cell Proliferation assay, following the manufacturer's recommendations. The results of cell viability for each condition were normalised by the control, which consisted of untreated cells, exposed to vehicle only. Two independent experiments were performed, and each condition was run in triplicate. For the apoptosis assay, SKOV3 cells were seeded in 96-well white flat bottom plates, at a density of 5000 cells/well in full media conditions, and were allowed to attach for 24 h at 37 ˚C in a humidified atmosphere of 5% CO_2. Cell media were then changed to serum-free RPMI-1640. After overnight serum starvation, cells were incubated with 100 µg/mL ApoA1 for 12 h and 48 h. Caspase 3/7 activation was measured using the ApoTox-Glo™ triplex assay, following manufacturer's instructions. Caspase 3/7 activity was normalised to cell viability obtained for each condition. The results of caspase 3/7 activation were compared with the untreated control cells, exposed to vehicle only. Two independent experiments were performed, and each condition was run in triplicate.

The effect of the ApoA1 mimetic peptide 4F on mitochondrial viability was investigated in SKOV3, OVCAR3 and CAOV3 cell lines. The toxicity of this peptide for non-neoplastic OSEC2 cells was also assessed. SKOV3, OVCAR3 and CAOV3 cells were seeded in 96-well plates, at a density of 5000 cells/well in full media conditions. Cells were allowed to attach for 24 h at 37 ˚C in a humidified atmosphere of 5% CO_2. Cells were then kept in serum-free media and after overnight serum starvation, they were incubated with increasing concentrations of ApoA1 mimetic peptide (0.5 – 100 µg/mL)[19] for 48 h. Similarly, OSEC2 cells were seeded at a density of 5000 cells/well, in 96-well plates, in full media conditions. Cells were allowed to attach for 24 h at 33 ˚C in a humidified atmosphere of 5% CO_2. Cell media were changed to serum-free RPMI-1640 and after overnight serum starvation, cells were incubated with 100 µg/mL ApoA1 mimetic peptide for 48 h. Mitochondrial viability was measured by CellTiter 96® AQueous One Solution Cell Proliferation assay, following the manufacturer's recommendations. The results of cell viability for each condition were normalised by the viability of untreated

[19] 100 µg/mL of ApoA1 corresponds to approximately 3.5 µM of protein (ApoA1 molecular weight: 28300 g/mol), while 100 µg/mL of the ApoA1 mimetic peptide corresponds to 43 µM of peptide (4F peptide molecular weight: 2310.62 g/mol).

cells, exposed to vehicle only. Two independent experiments were performed for each cell line; each condition was run in triplicate.

4.3.5. 3D tumour spheroid invasion assay

The effect of ApoA1 exposure on the ability of SKOV3 cells to invade the extracellular matrix was investigated in a 3D tumour spheroid invasion assay. The assay protocol was adapted from Vinci et al (2015). Briefly, SKOV3 cells were cultured in ultra-low attachment round bottom 96-well plates, at a density of 5000 cells/well in full media conditions. Cells were kept at 37 ˚C in a humidified atmosphere of 5% CO_2. After 3 days, the spheroids were serum-starved for 12 h. After this period of serum starvation, the spheroids were gently embedded in a mixture with equal volumes of serum-free RPMI-1640 and cold liquid growth factor reduced, phenol red-free, basement membrane Matrigel® matrix. Low concentration serum (0.3%) was added to stimulate invasion. ApoA1 was added to the mixture at a concentration of 300 µg/mL, while untreated spheroids exposed to vehicle only were used as controls. The invasion of extracellular matrix was assessed daily for 4 days, using an inverted microscope with a 4X or 10X objective, depending on the size of invaded area. Quantitative image analysis was performed with *ImageJ* software (*National Institute of Health*, MD, USA). The invasion area was normalised by the spheroid area at time=0. Two independent experiments were performed, each condition was run in triplicate.

4.3.6. Western blotting

For western blot analysis SKOV3 cells were seeded in 12-well plates, at a density of 60000 cells/well and allowed to attach during 24 h at 37 ˚C in a humidified atmosphere of 5% CO_2. Cells were serum-starved overnight and then treated with ApoA1 mimetic peptide at a concentration of 50 or 100 µg/mL, for 12 h and 24 h in serum-free conditions, before cell lysis for 20 min on ice. Briefly, cells were washed with PBS prior to collection in RIPA buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail II at the manufacturers' recommended concentrations.

Total protein concentration in the whole lysate was determined using the BCA Protein Assay Kit according to manufacturer's recommendations. Lysates were incubated at 100 ˚C for 10 minutes and then separated into 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred onto PVDF membranes. Membranes were blocked with 5% BSA in TBST buffer. Primary antibodies were diluted in 5% BSA in TBST buffer as follows: 1:6000 dilution for the calnexin antibody; 1:2000 dilution for both phospho-p44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}) antibody and p44/42 MAPK (ERK1/2) antibody; 1:1000 dilution for both the phospho-Akt Ser^{473} antibody and the pan Akt antibody. Incubation with primary antibodies was performed at 4 ˚C overnight. HRP-conjugated secondary antibodies were employed at a 1:5000 dilution in 1% BSA in TBST buffer, for incubation for 2h at room temperature. Proteins were detected using Immobilon™ Western Chemiluminescent HRP Substrate system and developed on X-ray film using Kodak SRX2000 (NY, USA) developer machine. Quantitative densitometry was performed using *ImageJ* software (*National Institute of Health*, MD, USA).

4.3.7. *In vitro* cisplatin sensitisation assay

In order to investigate the ability of the ApoA1 mimetic peptide to sensitise ovarian cancer cells to platinum-based chemotherapy, cell lines were co-incubated with different concentrations of the peptide and cisplatin, and mitochondrial viability was evaluated for each condition. SKOV3, OVCAR3 and CAOV3 cells were seeded in 96-well plates, at a density of 5000 cells/well in full media conditions. Cells were allowed to attach for 24 h at 37 ˚C in a humidified atmosphere of 5% CO_2. Cells were then exposed to the ApoA1 mimetic peptide and cisplatin in full media conditions for 72 h. Concentrations of the ApoA1 mimetic peptide ranged from 50 to 150 µg/mL, while cisplatin concentrations varied according to the sensitivity of each cell line. Cisplatin concentration ranged from 10 to 20 µM for SKOV3 cells, 1 to 5 µM for OVCAR3 cells and 2.5 to 5 µM for CAOV3 cells. Mitochondrial viability was determined by the CellTiter 96® AQueous One Solution Cell Proliferation assay, following the manufacturer's recommendations. The results for each condition were normalised by the control, which

consisted of untreated cells. Mitochondrial viability of cells incubated with the peptide in combination with cisplatin was compared with the mitochondrial viability of cells exposed to cisplatin only. Two independent experiments were performed, including four replicates per condition.

4.3.8. *In ovo* chicken chorioallantoic membrane assay

Fertilized brown chicken eggs were supplied by *Henry Stewart & Co. Ltd.* (Norfolk, UK). All the procedures involving chicken embryos were performed in agreement with Community and national legislation (Directive 2010/63/EU; Animals Scientific Procedures Act 1986) and also in agreement with the Basel Declaration. The experimental protocol received prior approval by the competent National Authority (project licence reference: PPL 70/7997; personal licence reference: I1D60DB1A) and by Imperial College's Animal Welfare and Ethical Review Body.

The method for implanting cancer cells on the chicken chorioallantoic membrane (CAM) was adapted from a previously described protocol (Sys et al., 2013), after in-house optimization (Antony et al., 2016). Briefly, fertilized eggs were incubated at 37.5 °C with 50% relative humidity for 3 days. On embryonic day 3, under sterile conditions, a small hole was pierced through the egg's taglion using a 19-gauge needle and 2-3 mL of albumen were removed to decrease the volume inside the egg and to ensure that the CAM is not damaged when the egg shell is opened. Subsequently, a 1 cm diameter window was opened on the egg's shell to expose the CAM. The window was covered with sterile film and the eggs were incubated at 37.5 ˚C with 50% relative humidity for 6 days. At embryonic day 9, xenografts were prepared by suspending 1×10^6 green fluorescent protein (GFP)-transduced SKOV3 cells in 100 µL of cold liquid Matrigel®. ApoA1 mimetic peptide and cisplatin were added to the cell suspension, at a concentration of 100 µg/mL and 15 µM, respectively. The compounds were added separately (peptide only, cisplatin only) or in combination (peptide plus cisplatin). Additionally, untreated cells exposed to vehicle were used as control. A medium to large blood vessel was then gently bruised using autoclaved round-bottom glass rods and the xenografts were topically inoculated onto this area. After inoculation, the egg shell

window was covered with sterile tape and the eggs were placed back in the incubator, at 37.5 ˚C with 50% relative humidity for 7 days. Tumour dimensions were quantified at embryonic day 16 using Zeiss SteREO Discovery V8 microscope and the Zen 2.0 blue edition software (Oberkochen, Germany).

4.3.9. Quantification of auto-antibodies towards high density lipoprotein and apolipoprotein A1

In order to investigate potential differences on the levels of auto-antibodies towards HDL and ApoA1 in women suffering from malignant and benign ovarian disease and evaluate how the levels of such antibodies compare with the levels observed in healthy women, we performed an exploratory clinical study. This clinical investigation was performed in accordance with the principles stated in the Declaration of Helsinki. The protocol was approved by *Instituto Português de Oncologia de Lisboa, Francisco Gentil, EPE*. Patients and volunteers were included after voluntarily giving their written informed consent. A blood sample (2 mL) was collected after diagnosis and prior to surgery, into tubes with no anticoagulant.

Serum anti-HDL and anti-ApoA1 IgG antibodies were quantified by in-house developed ELISA methods, as described by Batuca and collaborators (Batuca et al., 2007). Anti-HDL IgG antibodies were presented as a percentage of the control, while the anti-ApoA1 IgG antibodies were presented as μg/mL.

4.3.10. Statistical analysis

Statistical analysis was performed using GraphPad®Prism version 5.0 (*GraphPad Software Inc*., CA, USA). Cell viability is presented as a percentage, while caspase activation, relative invasion, western blot densitometry and xenografts area were presented as fold change from control. Data were analysed by two-way ANOVA, *Kruskal-Wallis* test, *Mann-Whitney* U test or *Student's* t test, as appropriate; a p value< 0.05 was considered significant.

4.4. Results

4.4.1. Apolipoprotein A1 and the 4F mimetic peptide decrease the viability of ovarian cancer cells

In order to investigate whether ApoA1 extracted from human plasma affects ovarian cancer cells viability, we analysed the effect of ApoA1, added to the extracellular media, on the viability of SKOV3 cells. These assays were performed in serum-free conditions to isolate the effect of human ApoA1, avoiding interferences with bovine ApoA1 and the variety of growth factors present in serum for cell media supplementation. Under these conditions, treatment with 100 µg/mL ApoA1 reduced the viability of SKOV3 cells by 28% (Figure 21, Panel A; *Kruskal-Wallis* test with *Dunn's* Multiple Comparison post-test; $p < 0.05$). This effect of ApoA1 in SKOV3 cells viability was independent of caspase 3/7 activation, as observed after 12 h and 48 h of incubation with ApoA1 (Figure 21, Panel B; two-way ANOVA; $p > 0.05$).

The ApoA1 mimetic peptide was tested in three different ovarian cancer cell lines, SKOV3, OVCAR3 and CAOV3 cells. The viability of cells treated with 100 µg/mL 4F was reduced by 28%, 40% and 20%, for SKOV3, OVCAR3 and CAOV3 cells respectively (Figure 22; Panels A, B and C; *Kruskal-Wallis* test with *Dunn'*s multiple comparison post-test; $p < 0.001$ for all cell lines). The peptide at a concentration of 50 µg/mL also reduced the viability of OVCAR3 and CAOV3 cells (Figure 22; Panels B and C; *Kruskal-Wallis* test with *Dunn'*s multiple comparison post-test; $p < 0.05$ for both cell lines). Importantly, the ApoA1 mimetic peptide does not affect the viability of non-neoplastic ovarian surface epithelial OSEC2 cells (Figure 22; Panel D; Mann-Whitney U test, $p > 0.05$).

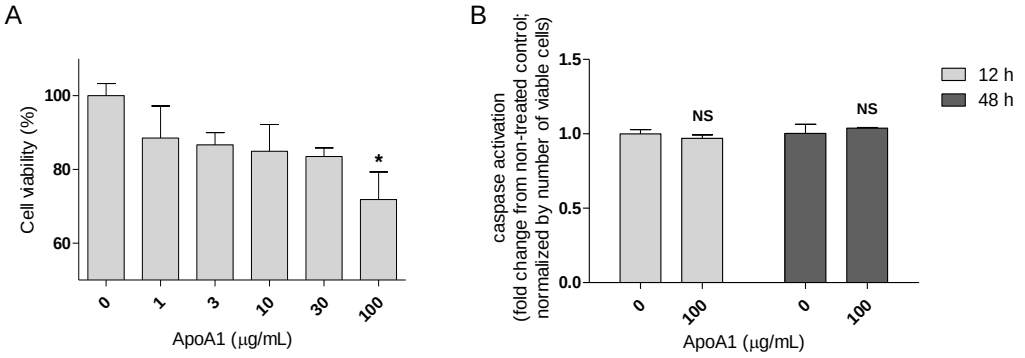

Figure 21 Apolipoprotein A1 decreases the viability of ovarian cancer cells without affecting caspase activation. *Panel A: SKOV3 cells were incubated with human apolipoprotein A1 (ApoA1) in the extracellular media, up to a concentration of 100 µg/mL in serum-free conditions for 48 h. Untreated cells were exposed to vehicle only. ApoA1 treatment significantly decreased the viability of SKOV3 cells (Kruskal-Wallis test with Dunn's multiple comparison post-test; p< 0.05). Panel B: SKOV3 cells were incubated with 100 µg/mL ApoA1 for 12 h (light grey bars) and 48 h (dark grey bars). Caspase 3/7 activation was quantified and normalised by the number of viable cells. No differences were observed in caspase activation between untreated cells and cells exposed to ApoA1. Differences were considered significant if p< 0.05.*

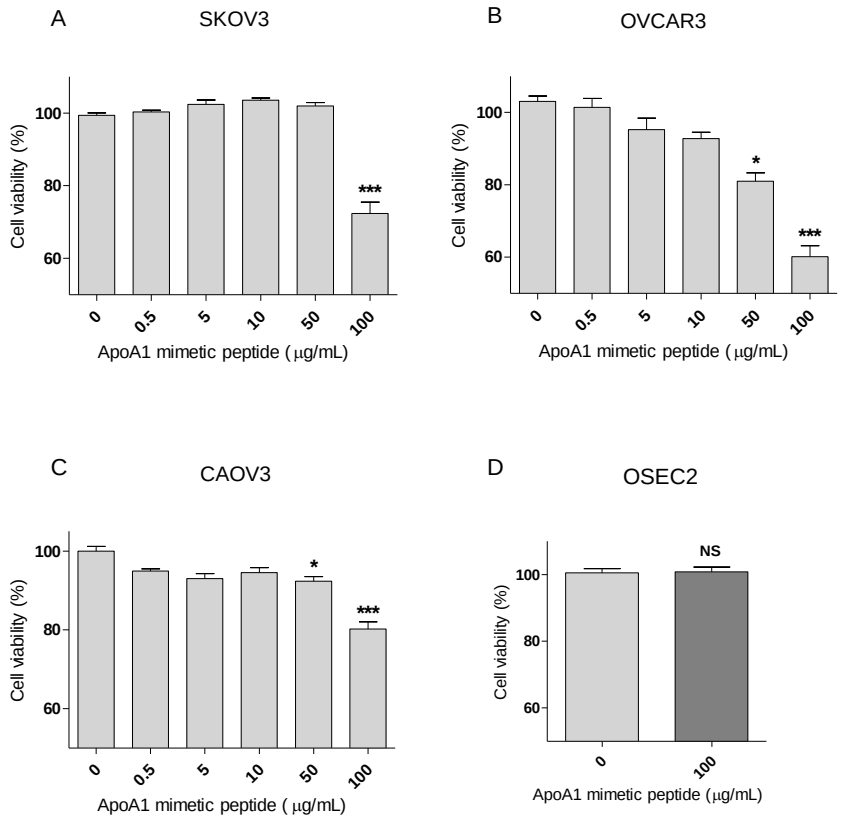

Figure 22 The apolipoprotein A1 mimetic peptide decreases the viability of ovarian cancer cells without affecting non-neoplastic ovarian cells. *SKOV3 (Panel A), OVCAR3 (Panel B) and CAOV3 (Panel C) cells were incubated with the apolipoprotein A1 (ApoA1) mimetic peptide 4F in the extracellular media up to a concentration of 100 µg/mL, in serum-free conditions for 48 h. Untreated cells were exposed to vehicle only. Exposure to the highest concentration of the peptide decreased the viability of all the three cell lines (Kruskal-Wallis test with Dunn's multiple comparison post-test; p< 0.001). The peptide at 50 µg/mL also decreased the viability of OVCAR3 and CAOV3 cells (p< 0.05). Immortalized non-neoplastic human ovarian surface epithelia cells (OSEC2; Panel D) were exposed to the highest concentration of peptide (100 µg/mL) used in the viability assay for the ovarian cancer cell lines. At this concentration the peptide did not affect the viability of OSEC2 cells. Differences were considered significant if p< 0.05.*

4.4.2. Apolipoprotein A1 reduces the ability of ovarian cancer cells to invade the extracellular matrix

The 3D tumour spheroid invasion assay was performed in spheroids of SKOV3 cells unexposed (control) and exposed to 300 µg/mL ApoA1 (Figure 23). Spheroids were embedded in Matrigel® matrix. Invasion of extracellular matrix was assessed daily, for 4 days (96 h), and the invasion area for each day was normalised by the spheroid area at time=0. As shown in Figure 23, ApoA1 treatment significantly decrease the ability of SKOV3 cells to invade extracellular matrix, after 72 h (Two-way ANOVA; control: 2.97 ±

0.06; ApoA1: 1.76 ± 0.09; $p < 0.001$) and 96 h of treatment (control: 2.93 ± 0.28; ApoA1: 2.11 ± 0.13; $p < 0.05$).

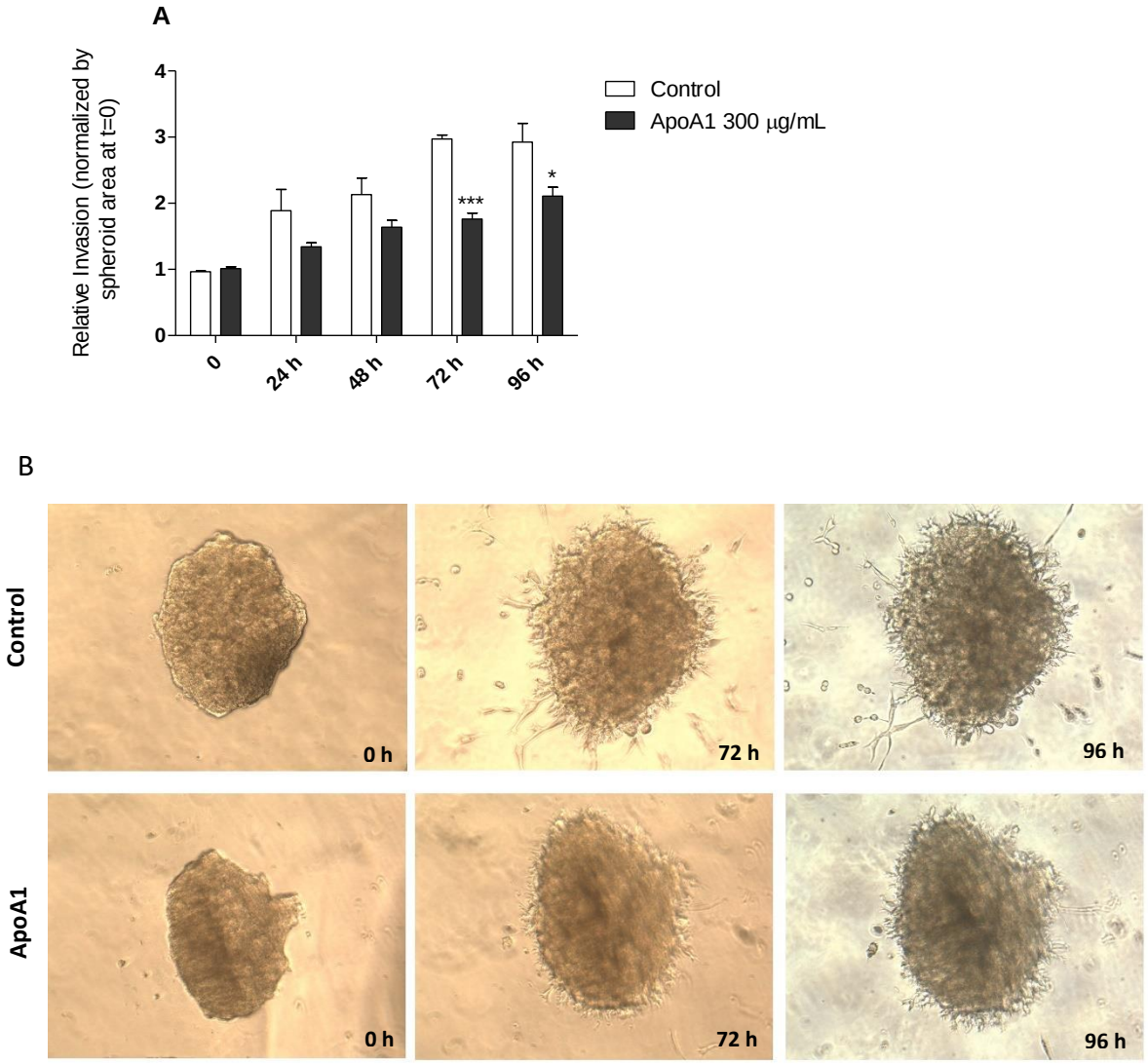

Figure 23 Apolipoprotein A1 decreases the ability of ovarian cancer cells to invade the extracellular matrix*. The 3D tumour spheroid invasion assay was performed in SKOV3 spheroids exposed to apolipoprotein A1 (ApoA1) at a concentration of 300 μg/mL, and in unexposed spheroids (control). Invasion of extracellular matrix was assessed for up to 96 h. Exposure to ApoA1 significantly decreased the ability of SKOV3 cells to invade the extracellular matrix after 72 h (Two-way ANOVA; p< 0.001) and 96 h (p< 0.05). On Panel A, the relative invasion normalised by the spheroid area at time=0 is plotted for ApoA1-treated and untreated spheroids. On Panel B, it is shown two spheroids, representatives of the ApoA1-treated and control conditions, at the beginning of the experiment (time=0) and during the course of experiment (72 h and 96 h). Differences were considered significant if p< 0.05.*

4.4.3. Apolipoprotein A1 mimetic peptide strongly affects Akt phosphorylation

We investigated whether the incubation with the ApoA1 mimetic peptide would affect Akt and ERK signalling in SKOV3 cells. As demonstrated in Figure 24, SKOV3 cells treated with 100 µg/mL ApoA1 mimetic peptide showed a significant decrease in Akt Ser^{473} phosphorylation after 12 h (Figure 24, Panels A and C; two-way ANOVA; 0.52 ± 0.08; $p< 0.01$) or 24 h (0.48 ± 0.10; $p< 0.01$) of exposure. Despite not reaching statistical significance, a trend for increased phosphorylation of ERK1/2 at Thr^{202}/Tyr^{204} upon treatment with the peptide was also observed. Nevertheless, this effect on ERK phosphorylation was more pronounced after 12 h of exposure, being reduced after 24 h of treatment (Figure 24, Panels A and E). No significant changes were observed regarding pan Akt levels or total ERK1/2 (Figure 24, Panels B, D and F). These results support that the ApoA1 mimetic peptide preferentially affects Akt signalling compared to ERK1/2 (Figure 24, Panels A and C); this observation prompted the evaluation on the effect of this peptide on cisplatin sensitisation.

4.4.4. Apolipoprotein A1 mimetic peptide sensitises ovarian cancer cells to cisplatin

The results presented in Figure 25 show that, in full media conditions, the ApoA1 mimetic peptide sensitises all the three ovarian cancer cell lines tested to cisplatin. For example, the viability for SKOV3 cells exposed to 15 µM cisplatin, with no peptide, was approximately 58%; while SKOV3 cells exposed to 15 µM cisplatin in combination with increasing concentrations of the peptide – 50, 100 and 150 µg/mL – presented a cell viability of 50% (Two-way ANOVA; $p< 0.05$), 33% ($p< 0.001$) and 16% ($p< 0.001$), respectively. Likewise, the viability for OVCAR3 cells was significantly reduced upon concomitant treatment with cisplatin and the ApoA1 mimetic peptide. For this cell line, treatment with 3 µM cisplatin resulted in approximately 90% cell viability; however, co-incubation with 50, 100 or 150 µg/mL of 4F peptide caused a significant decrease in cell viability, to 75% ($p< 0.05$), 62% ($p< 0.001$) and 43% ($p< 0.001$), respectively. Similarly, CAOV3 cells treated with 2.5 µM cisplatin reached a cell viability of 44%; when cells were exposed to 2.5 µM cisplatin in combination with 100 and 150 µg/mL of the ApoA1

mimetic peptide the viability significantly decreased to 24% ($p< 0.001$) and 5% ($p< 0.001$), respectively (Figure 25).

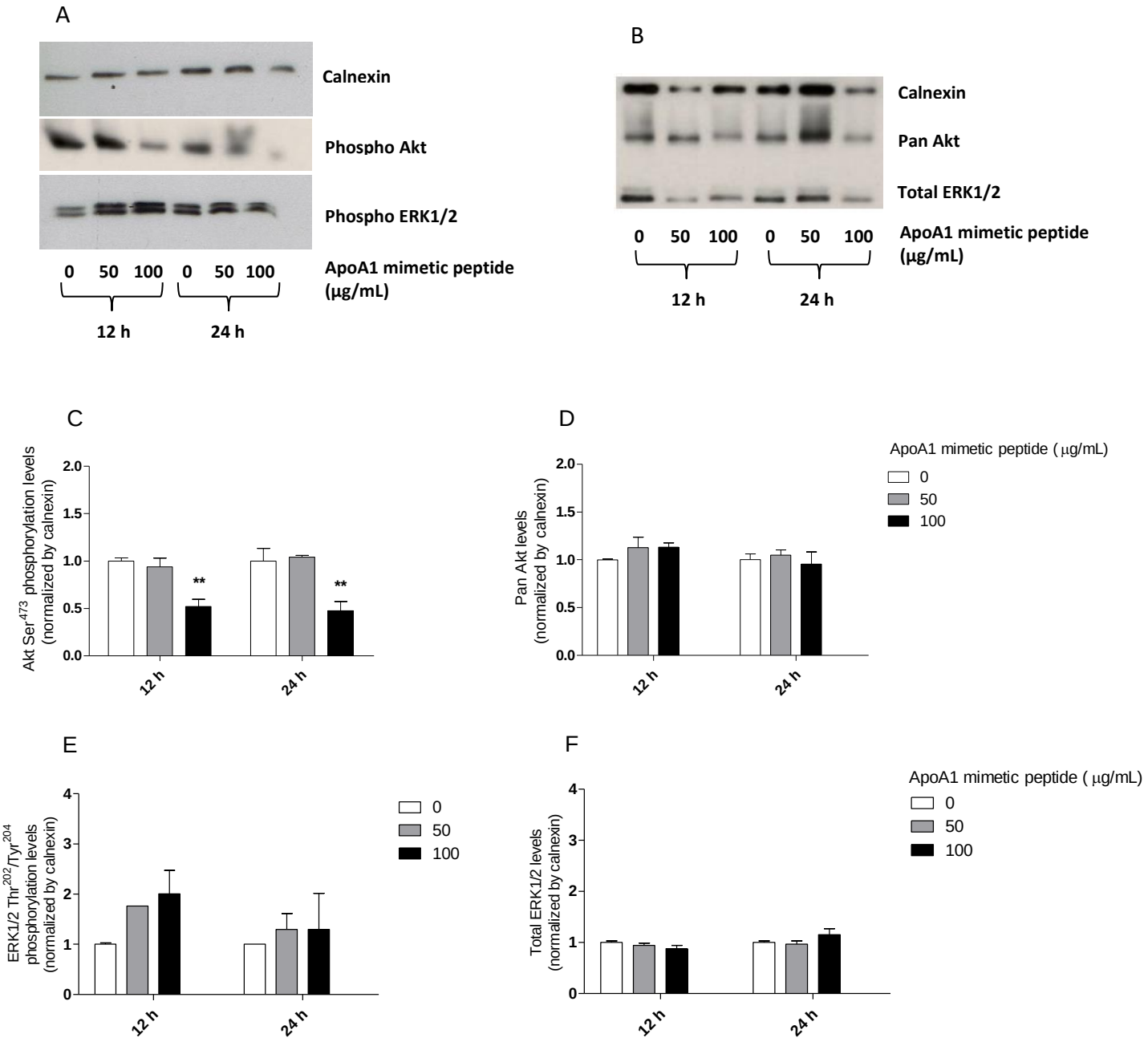

Figure 24 Apolipoprotein A1 mimetic peptide strongly suppresses Akt signalling in ovarian cancer cells. *After overnight serum starvation, SKOV3 cells were treated with the apolipoprotein A1 (ApoA1) mimetic peptide (50 µg/mL or 100 µg/mL). Untreated SKOV3 cells were used as control. Cell lysates were collected 12 h or 24 h after treatment and subjected to western blot analysis. Calnexin was the loading control for these experiments. Panel A: western blot for phospho Akt (Ser^{473}) and phospho ERK1/2 (Thr^{202}/Tyr^{204}); Panel B: western blot for pan Akt and total ERK1/2; Panel C: densitometry for Akt Ser^{473} phosphorylation; Panel D: densitometry for Pan Akt; Panel E: densitometry for ERK1/2 Thr^{202}/Tyr^{204} phosphorylation; Panel F: densitometry for total ERK1/2. The ApoA1 mimetic peptide strongly decreased phosphorylation of Akt at Ser^{473} after 12 h (two-way ANOVA; p< 0.01) and 24 h (p< 0.01) of exposure with the highest concentration of peptide (100 µg/mL). Differences were considered significant if p< 0.05.*

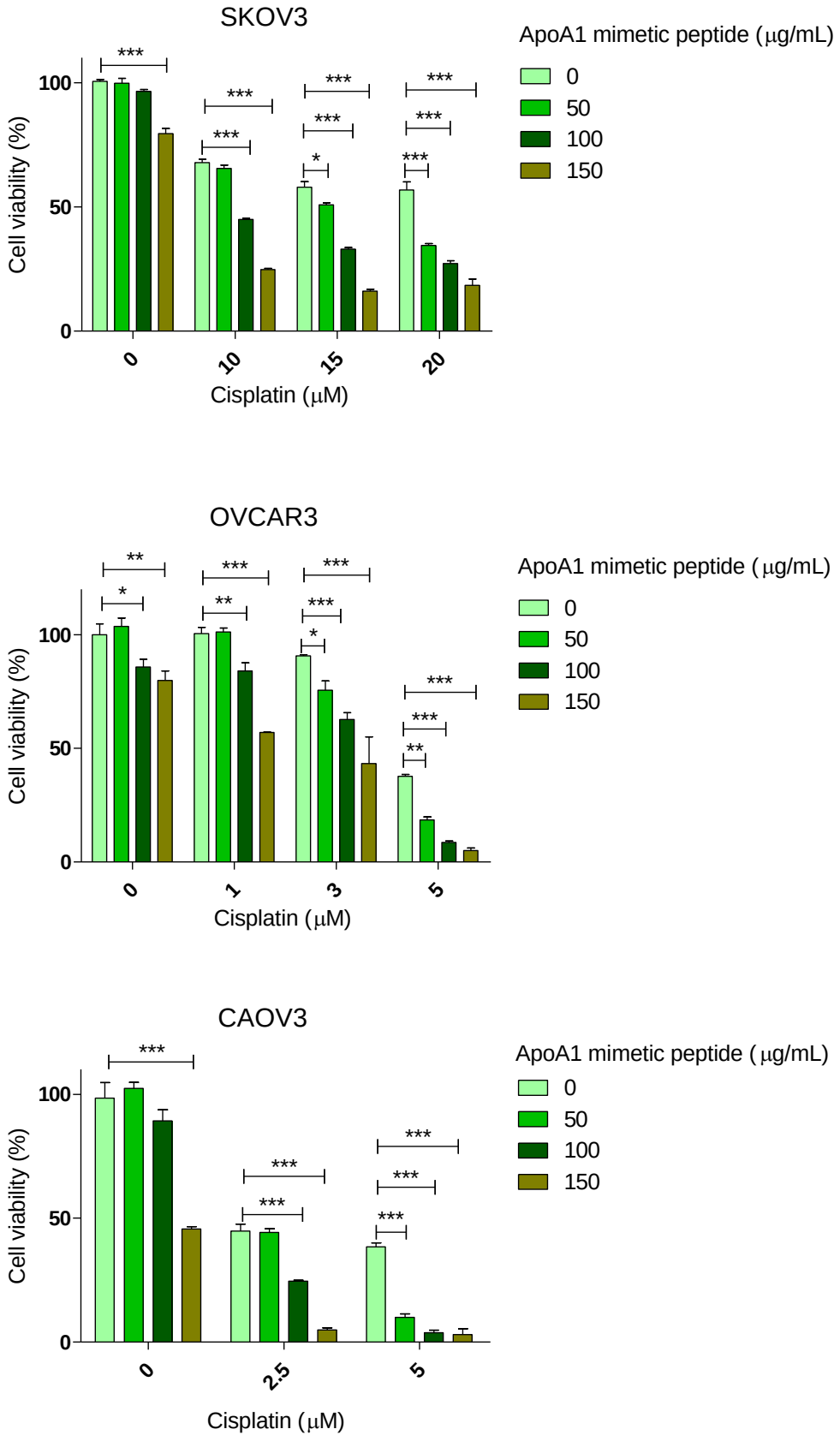

Figure 25 Apolipoprotein A1 mimetic peptide sensitises ovarian cancer cells to cisplatin. *SKOV3, OVCAR3 and CAOV3 cells were co-incubated with increasing concentrations of apolipoprotein A1 (ApoA1) mimetic peptide, up to 150 μg/mL, and increasing concentrations of cisplatin, according to the sensitivity of each cell line. Exposure to the peptide and cisplatin was performed in full media conditions. After 72 h, mitochondrial cell viability was assessed. Two-way ANOVA was performed as statistical test. *p< 0.05; **p< 0.01; ***p<0.001.*

4.4.5. The effect of apolipoprotein A1 mimetic peptide on cisplatin sensitisation was investigated in the chicken chorioallantoic membrane model

To investigate the effect of the ApoA1 mimetic peptide in terms of cisplatin sensitisation in a biologically relevant system, we inoculated GFP-transduced SKOV3 cells xenografts on the chicken CAM model. Xenografts were exposed *in ovo* to cisplatin and ApoA1 mimetic peptide, separately and in combination.

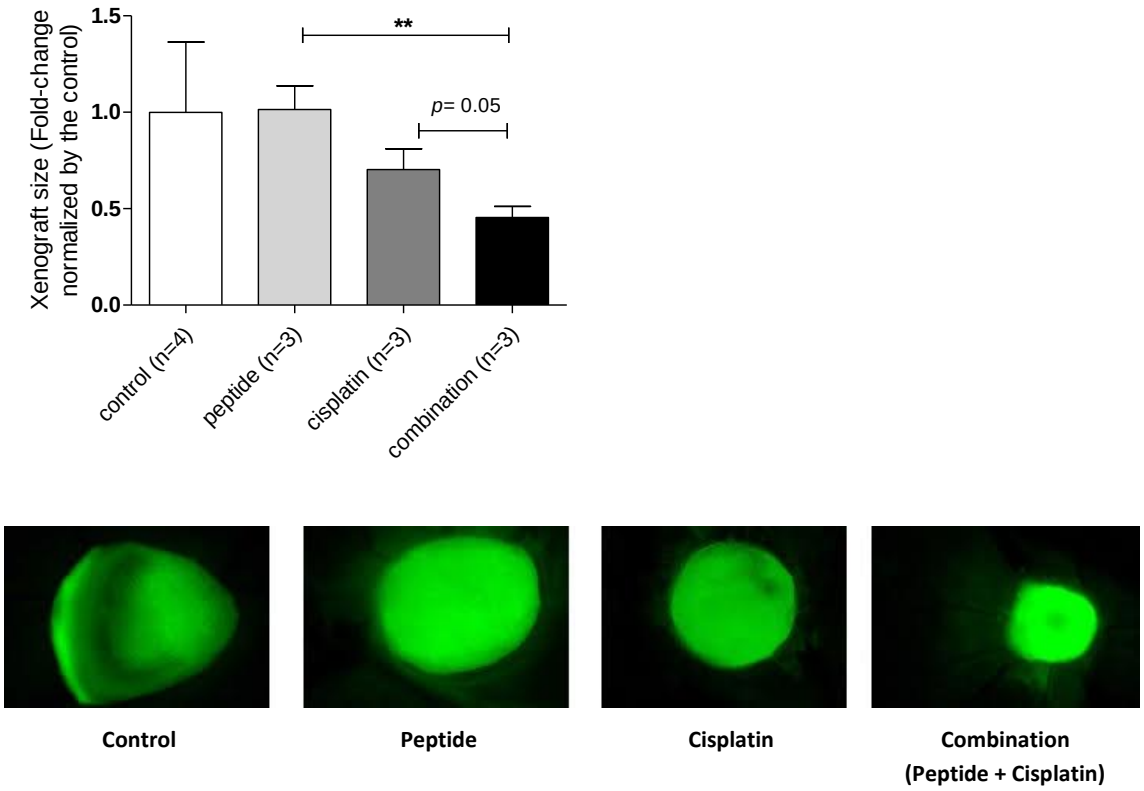

Figure 26 Apolipoprotein A1 mimetic peptide sensitises ovarian cancer cells to cisplatin in the biologically relevant in ovo model. *SKOV3 xenografts were topically inoculated on the chicken chorioallantoic membrane (CAM), left untreated (control) or treated with the apolipoprotein A1 (ApoA1) mimetic peptide (100 µg/mL), cisplatin (15 µM) or with the combination of both (100 µg/mL peptide plus 15 µM cisplatin). Xenograft size was calculated as the area of the fluorescent tumour relative to background. Student's t test was performed after testing the data normality by the Shapiro-Wilk normality test. Differences were considered significant if p< 0.05. **p< 0.01.*

The embryo mortality in this assay was extremely high (approximately 70%) which was probably related to the incubator malfunctioning, affecting temperature and humidity controls. Despite the high mortality, the size of the xenografts exposed to the peptide in combination with cisplatin was significantly smaller than the size of the xenografts

exposed to the peptide alone (Figure 26; *Student's* t test; peptide treated: 1.0 ± 0.12; combination: 0.45 ± 0.06; *p*= 0.007). Furthermore, we could also observe a trend for smaller size on the xenografts exposed to the peptide in combination with cisplatin, compared to cisplatin alone (cisplatin treated: 0.70 ± 0.11; combination: 0.45 ± 0.06; *p*= 0.05). Therefore, the *in ovo* findings seem to substantiate the *in vitro* results regarding the peptide ability to induce cisplatin sensitisation.

4.4.6. The levels of auto-antibodies towards high density lipoprotein and apolipoprotein A1 were investigated in women suffering from ovarian disease

The levels of anti-HDL and anti-ApoA1 IgG antibodies were quantified in 19 women with ovarian cancer, in 17 women with benign ovarian disease and also in 54 healthy volunteers. No significant differences were found between groups regarding the levels of anti-HDL antibodies (Figure 27; Panel A; *Kruskal-Wallis* test with *Dunn's* multiple comparison post-test, *p*> 0.05) or anti-ApoA1 antibodies (Figure 27; Panel B; *p*> 0.05).

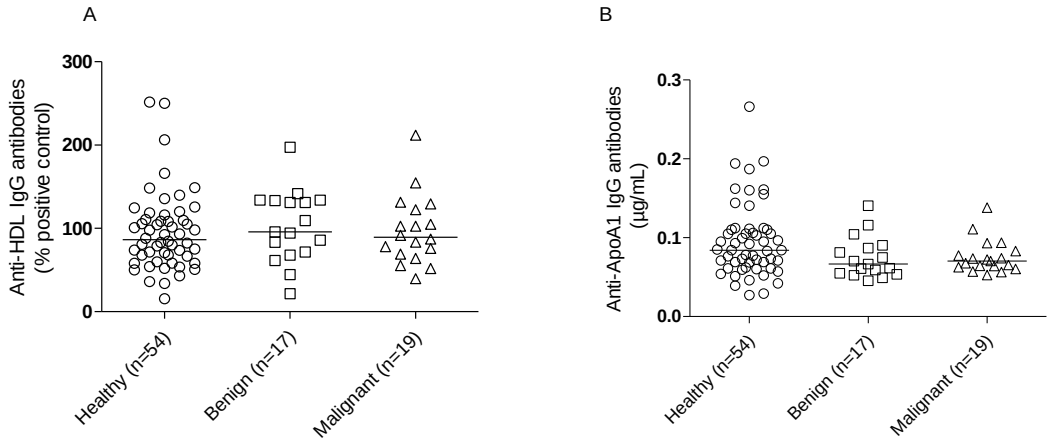

Figure 27 Auto-antibodies towards high density lipoprotein and apolipoprotein A1 in women suffering from malignant and benign ovarian disease comparatively to healthy women. *The serum levels of auto-antibodies towards high density lipoprotein (HDL; Panel A) and apolipoprotein A1 (ApoA1; Panel B) were quantified in women with malignant and benign ovarian disease and also in healthy volunteers. Data are presented as median [IQR]. Kruskal-Wallis test with Dunn's multiple comparison post-test was employed as statistical test. Differences were considered significant if p< 0.05.*

4.5. Discussion

In the current study, we demonstrated the effect of ApoA1 and the ApoA1 mimetic peptide 4F (Ac-$F^{3,14}$18A-NH_2) as suppressors of ovarian tumorigenesis. The anti-tumorigenic properties of both ApoA1 and ApoA1 mimetic peptides have been recently explored in different types of malignancies, including melanoma, lung cancer (Zamanian-Daryoush et al., 2013; Zamanian-Daryoush and DiDonato, 2015), colorectal cancer (Gkouskou et al., 2016) and ovarian cancer (Su et al., 2010; Gao et al., 2011; Ganapathy et al., 2012). The main novelties reported in this Chapter, which distinguish this work from previous findings, are the ability of human ApoA1 to suppress the invasive phenotype of ovarian cancer cells, and the chemosensitisation induced by the ApoA1 mimetic peptide.

ApoA1 is best known for its role in the regulation of cholesterol homeostasis and in reverse cholesterol transport (Heinecke, 2012; Kingwell et al., 2014), however this anti-atherogenic apolipoprotein has also a crucial importance in ovarian physiology and in the regulation of steroidogenesis (Enk et al., 1986; Bogan and Hennebold, 2010; Sriraman et al., 2010). It is known that the levels of ApoA1 are remarkably reduced in serum and in the ovarian tissues of women suffering from ovarian cancer (Wegdam et al., 2014). For this reason, ApoA1 was included in a panel of five serum biomarkers commercially available for assessing the likelihood of malignancy of an ovarian mass, prior to surgery (Nolen and Lokshin, 2013). Moreover, it is recognised that higher serum ApoA1 levels are associated with a better prognosis for ovarian cancer patients (Stavnes et al., 2014) and also for patients suffering from other tumours, such as nasopharyngeal carcinoma, renal cell carcinoma, breast cancer, colorectal and lung cancer (Walter et al., 2012; Cheng et al., 2015; Luo et al., 2015; Guo et al., 2016; Lin et al., 2017; Sirniö et al., 2017). *In vivo* and *in vitro* evidence also supports the hypothesis of an anti-tumorigenic effect of ApoA1. For instance, overexpression of human ApoA1 decreased tumour development in rodent models of melanoma and lung cancer, while ApoA1 knockout had the opposite effect in the same animal models (Zamanian-Daryoush et al., 2013). Importantly, subcutaneous injection of human ApoA1 decreased tumour burden, prevented metastatic growth and improved survival of mice previously inoculated with melanoma cells, after the establishment of palpable tumours and metastasis (Zamanian-

Daryoush et al., 2013), arguing for the potential therapeutic applications of ApoA1 for cancer at more advanced stages. Moreover, ApoA1 ablation exacerbated the pathological and inflammatory features and also the proliferative index associated with colitis-induced colorectal carcinogenesis in rodents (Gkouskou et al., 2016). Relatively to ovarian malignancies, overexpression of human ApoA1 also decreased tumour volume, prevented metastatic growth and increased survival in a transgenic mice model of ovarian cancer (Su et al., 2010). The anti-tumorigenic mechanisms attributed to ApoA1 were related to the modulation of the tumour microenvironment, the inhibition of tumour neoangiogenesis, the regulation of inflammatory signalling (*eg.* through the signal transducer and activator of transcription 3 (STAT3) pathway) and to decreased levels of pro-invasion factors, such as MMP-9 (Zamanian-Daryoush et al., 2013; Zamanian-Daryoush and DiDonato, 2015; Gkouskou et al., 2016). The effect of ApoA1 in STAT3-MMP-9 is particularly interesting since aggressive ovarian cancer cells, such as SKOV3, express high levels of MMP-9 metalloproteinase through phosphorylation of STAT3 at Tyr^{705} (Jia et al., 2017). Therefore, ApoA1-mediated STAT3 signalling inhibition with consequent decreased expression of MMP-9 is consistent with our observation of decreased invasion of extracellular matrix in SKOV3 spheroids exposed to ApoA1. Additionally, ApoA1 was demonstrated to decrease the viability of gastric adenocarcinoma cells *in vitro,* through abrogating proliferation induced by LPA (Yeh et al., 2016), a pro-inflammatory pro-carcinogenic lysophospholipid (reviewed in *Introduction*, *Section 2.1.3*). It is important to highlight that LPA, whose plasma levels are increased in approximately 90% of all ovarian cancer patients (Mills and Moolenaar, 2003; Bast et al., 2009), is crucially involved in the modulation of several intracellular pathways contributing to ovarian cancer pathophysiology (Fang et al., 2002; Ottevanger, 2017).

Besides ApoA1, some ApoA1 mimetic peptides have also been explored for their anti-tumorigenic properties. ApoA1 mimetics are synthetic amphipathic α-helical peptides with only 18 aminoacids, that can mimic the structure of an ApoA1 α-helix and replicate, at least to some extent, the functional properties of ApoA1 (Datta et al., 2001; Leman et al., 2014). In fact, these small peptides are able to effectively mimic several aspects of ApoA1 functionality, such as interaction with phospholipids and ABC transporters,

promotion of cholesterol efflux from macrophages, inhibition of monocyte chemotaxis induced by oxidised LDL, stabilisation of the PON-1 enzyme and inhibition of the atherosclerotic process in the arterial wall (Datta et al., 2001; Xie et al., 2010; Mishra et al., 2013; Ying et al., 2013). However, there are some functional differences between ApoA1 and its mimetic peptides. For instance ApoA1 mimetic peptides show limited ability to activate the LCAT enzyme, unlike ApoA1 which can fully activate this enzyme (Datta et al., 2001); in contrast, ApoA1 mimetic peptides can bind to pro-inflammatory and oxidised lipids, including LPA, more effectively than ApoA1 (Van Lenten et al., 2008). Regarding the effect of ApoA1 mimetics in ovarian cancer, Su and collaborators (2010) reported the beneficial effect of two peptides, 4F and 5F (Ac-$F^{11,14,17}$18A-NH_2), in terms of tumour development *in vivo*, after subcutaneous and intraperitoneal injection of an epithelial ovarian cancer cell line in mice. The mechanisms implicated in this effect of ApoA1 mimetic peptides on ovarian cancer progression were probably related to the binding and sequestration of LPA and other inflammatory lipids, consequently leading to a reduction in cellular oxidative stress and inhibition of pro-angiogenic signalling (*eg*. HIF-1-dependendent transcription of VEGF) (Saunders et al., 2010; Su et al., 2010; Gao et al., 2011, 2012; Ganapathy et al., 2012). In regard to the anti-tumorigenic effects reported in the present work, the involvement of LPA sequestration seems plausible due to the high-affinity between 4F and LPA (Van Lenten et al., 2008; Su et al., 2010) and also considering that all the three ovarian cancer cell lines employed here, unlike normal ovarian epithelial cells, produce LPA as an autocrine/paracrine factor, for stimulation of proliferation, survival, angiogenesis and for the pro-carcinogenic modulation of tumour microenvironment (Hu et al., 2001; Chou et al., 2004; Lee et al., 2006; Thibault et al., 2014). Moreover, it is recognised that HIF-1-induced VEGF expression can lead to increased Akt phosphorylation at Ser^{473} in ovarian granulosa cells and in ovarian cancer cells, consequently leading to increased cell proliferation (Trinh et al., 2009; Irusta et al., 2010; Shiratsuki et al., 2016). Therefore, ApoA1 mimetic-mediated inhibition of HIF-1/VEGF signalling could also be involved on the inhibition of Akt phosphorylation reported in here, observed in ovarian cancer cells upon exposure to 4F. Additionally, it is known that cholesterol depletion in the membrane lipid rafts, promoted by both ApoA1 and the ApoA1 mimetic peptides, inhibits Akt signalling (Gaus et al., 2004; Beloribi-Djefaflia et al., 2016; Iqbal et al., 2016). Further studies must be conducted to

clarify the involvement and contribution of LPA sequestration or signalling modulation in lipid rafts for the effects mediated by ApoA1 and its mimetic peptides.

Importantly, an extremely large body of evidence demonstrates hyperactivation of PI3K/Akt kinases in several malignancies (Altomare and Testa, 2005; Gabra et al., 2008; Liu et al., 2009; Stronach et al., 2011; Cheung and Testa, 2013; Mabuchi et al., 2015; Hahne et al., 2016). Aberrant PI3K/Akt signalling contributes to several processes that are considered cancer hallmarks or that are related to these hallmarks, namely increased survival and inactivation of apoptotic mechanisms, uncontrolled cell cycle progression, inhibition of tumour suppressor proteins, increased neo-angiogenesis, induction of epithelial-mesenchymal transition, enhanced cell invasion, migration and metastatic growth (Altomare and Testa, 2005; Gabra et al., 2008; Hanahan and Weinberg, 2011; Stronach et al., 2011; Cheung and Testa, 2013; Hahne et al., 2016). Both receptors tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) can be upstream regulators of Akt signalling (Chou et al., 2004; Moritz et al., 2010; Saunders et al., 2010; McKie et al., 2012; Mabuchi et al., 2015; Oda et al., 2015; Riaz et al., 2016; Manning, 2017), for instance Gαi/o βγ proteins coupled to LPA receptors can lead to the activation PI3K and consequently to phosphorylation of Akt (Saunders et al., 2010; Riaz et al., 2016); another example is the opioid-binding protein cell adhesion molecule (OPCML), which exerts its tumour suppressor functions through regulation of specific RTKs leading to the abrogation of Akt phosphorylation in ovarian cancer cells (McKie et al., 2012). Moreover, significant crosstalk between RTKs and GPCRs, affecting the PI3K/Akt pathway, has been reported in ovarian granulosa cells and other tissues (Pyne et al., 2003; Gavi et al., 2006; Shepard et al., 2008; Oyesanya et al., 2010; Cai and Xu, 2013; Law et al., 2016). An example is the EGFR-LPA receptor crosstalk in human epithelial ovarian cancer cell lines (Oyesanya et al., 2010; Cai and Xu, 2013). Importantly, the PI3K/Akt signalling pathway not only contributes to ovarian cancer development and tumorigenesis but has also been implicated in the mechanism of chemoresistance of ovarian cancer cells to platinum and other drugs, such as taxanes and doxorubicin (Blagden and Gabra, 2009; Stronach et al., 2011; Cheraghchi-Bashi et al., 2015; Sasano et al., 2015; Brasseur et al., 2017). Therefore, targeting Akt signalling has become an appealing strategy for overcoming chemoresistance in ovarian malignancies (Gabra et

al., 2008; Blagden and Gabra, 2009; Stronach et al., 2011; Cheraghchi-Bashi et al., 2015; Gungor et al., 2015; Sasano et al., 2015). Here we demonstrated for the first time that an ApoA1 mimetic peptide, affecting Akt signalling, can be useful as a platinum-sensitising agent, enhancing the response to chemotherapy, although the *in ovo* CAM assay needs further optimization in order to definitively prove this effect in a biologically relevant system. The potential translational applications of these therapeutic approaches are particularly promising, considering that up to 85% of women with epithelial ovarian cancer who achieve full remission after 1st line chemotherapy will develop recurrent disease (Foley et al., 2013) and the 5-year survival rate for this disease is as low as 46% (Miller et al., 2016). Thus, the combination of conventional cytotoxic drugs with agents targeting the PI3K/Akt pathway can bring real benefits to those patients.

Considering the evidence discussed above on the role of ApoA1 in the cancer context, we investigated the levels of auto-antibodies towards HDL and ApoA1 in women with malignant and benign ovarian disease comparatively to the levels observed in healthy women. The antibodies towards HDL and ApoA1 are recognised as strongly pro-inflammatory, being able to activate inflammatory pathways through interaction with TLR2 and TLR4, consequently leading to increased expression of MMP-9 in vascular cells and activating PI3K/Akt signalling in cardiomyocytes (Chistiakov et al., 2016). Although it is not clear if this antibody-induced pathophysiological mechanism is relevant for ovarian cancer, it is known that ovarian tumours express higher levels of TLR2 and TLR4 than the normal ovarian surface epithelia (Zhou et al., 2009; Woods et al., 2011). However, there is a scarcity of studies focusing on these antibodies in malignant diseases. As far as we know, this is the first study investigating the levels of anti-HDL and anti-ApoA1 antibodies in women with malignant and benign ovarian disease. We have not found any differences on the levels of such antibodies among women with ovarian disease comparatively to healthy controls. A possible explanation for this observation would be the decreased levels of ApoA1 (*i.e.* decreased levels of the antigen) in ovarian cancer patients (Wegdam et al., 2014).

Here, it was demonstrated that the ApoA1 mimetic peptide does not affect the viability of normal OSEC2 cells at a dose of 100 μg/mL, which is an important observation

concerning the absence of toxicity towards non-cancerous cells, at least *in vitro,* and therefore a lower potential for off-target effects. A single oral dose of 4F peptide, corresponding to a peak plasma concentration of 0.01 µg/mL, was well tolerated in patients with coronary heart disease (Bloedon et al., 2008); higher doses were subcutaneously administered to mice, reaching a peak plasma concentration of 61 µg/mL (Navab et al., 2011). Further studies are needed to assess the safety of ApoA1 and its mimetic peptides at relevant doses for the anti-tumorigenic and chemosensitising effect. Additionally, it would be worth to investigate the potential of ApoA1 booster therapies for cancer treatment. Our team provided evidence for the ApoA1 booster effect of NVP and its phase I metabolite 2-OH-NVP (*cf.* Chapter 3 of the current dissertation; Marinho et al., 2014a), thus NVP, the 2-OH-NVP metabolite or an analogue would be an excellent starting point for exploring the usefulness of ApoA1 boosters in ovarian cancer therapy. Moreover, and besides its effect on ApoA1, NVP anti-proliferative properties have been studied before. In fact, *in vitro* exposure to NVP at concentrations up to 400 µM caused cell cycle arrest in different human cancer cell lines, including colorectal adenocarcinoma, hepatocellular carcinoma, breast cancer, astrocytoma, osteosarcoma, melanoma and prostate carcinoma cell lines (Mangiacasale et al., 2003; Sciamanna et al., 2005; Fang and Beland, 2013). Inhibition of the endogenous reverse transcriptase in cancer cells is thought to contribute to the NVP effect on cell proliferation, due to the potential oncogenic activity of the endogenous non-telomerase reverse transcriptase (Sciamanna et al., 2005; Sinibaldi-Vallebona et al., 2006; Chow et al., 2009). However NVP is also able to induce an anti-proliferative response in cancer cells with no reverse transcriptase activity (Fang and Beland, 2013), suggesting that possibly a more direct toxic mechanism might be underlying NVP effect in some cancer cells (Paemanee et al., 2017). As discussed before, NVP toxic reactions (reviewed in *Introduction*, *Section 3.2;* Marinho et al., 2014c) and its ApoA1 booster effect (*cf.* Chapter 1; Marinho et al., 2014a) are both predominant among female patients; these observations lead us to speculate if the sex-dependent dimorphic profile of toxicity and ApoA1 modulation might contribute to a better therapeutic outcome for women suffering from ovarian cancer. As far as we know, this approach has never been tested in ovarian cancer patients, however a case report demonstrated the efficacy of NVP in slowing cancer progression in an elderly female patient with advanced-stage

papillary thyroid carcinoma, who received the conventional treatment in association with 400 mg NVP daily for 7 months (Modoni et al., 2007); unfortunately, neither NVP pharmacokinetic profile nor ApoA1 levels were assessed in this patient, which prevents us from making further considerations on the mechanism behind this positive outcome. In conclusion, this work not only confirms the anti-tumorigenic properties of ApoA1 and ApoA1 mimetic peptides, but also supports the notion of these biomolecules as chemosensitising agents in ovarian cancer. Considering that the emergence of chemoresistance is a major challenge in the management of ovarian cancer (Foley et al., 2013; Palmirotta et al., 2017) and the dismal prognosis associated with this malignancy (Miller et al., 2016), the therapeutic use of ApoA1, ApoA1 mimetics or an ApoA1 booster, such as 2-OH-NVP or other NVP analogues, could have a real positive impact for ovarian cancer treatment. We believe this therapeutic approach is worth testing in the future.

FINAL CONSIDERATIONS

The research work described in the current dissertation was focused on the re-profiling of NVP, a widely prescribed antiretroviral, as an HDL modulator. Many studies have associated NVP treatment with beneficial changes in the lipid profile of HIV-infected patients, particularly regarding increases in HDL-cholesterol (Ruiz et al., 2001; van der Valk et al., 2001; Clotet et al., 2003; van Leth et al., 2004; Sankatsing et al., 2007; Floridia et al., 2009; Franssen et al., 2009; Podzamczer et al., 2011; 2014; Soriano et al., 2011; Strehlau et al., 2012; Arpadi et al., 2013). It has been reported that NVP ability to modulate HDL and ApoA1 is an intrinsic property of the drug and not a consequence of HIV replication control (Sankatsing et al., 2007; Franssen et al., 2009). Moreover, these NVP-associated changes might be translated into real clinical benefits, being associated with a significant reduction of atherosclerotic lesions (Maggi et al., 2011; Gleason et al., 2016). Importantly, induction of ApoA1 synthesis was recently recognised as one of the most promising strategies for long-term prevention of atherosclerosis (Gadkar et al., 2016). Despite the clinical interest in pharmacological approaches targeting ApoA1 and HDL, currently there are no drugs to effectively increase HDL-cholesterol levels or to improve HDL particle functionality. All the attempts to develop an ApoA1/HDL booster have failed due to toxicity or lack of clinical benefit (Table1). Based on these premises, NVP seemed to us an interesting starting point for the development of an HDL booster. Though intriguingly, NVP as a CAR agonist (Faucette et al., 2006) would probably not be the molecule responsible for the modulation of HDL-cholesterol observed upon NVP treatment, as CAR activation decreases the hepatic expression of ApoA1 and therefore the levels of HDL-cholesterol (Masson et al., 2008; Naik et al., 2013). Would be a NVP metabolite the responsible for the modulatory effect on HDL?

Moreover, NVP association with idiosyncratic skin rash and hepatotoxicity is a major drawback, preventing its re-profiling for other therapeutic areas. Our research team (Antunes et al., 2008, 2010a, 2010b; Caixas et al., 2012; Marinho et al., 2014c; Pinheiro et al., 2015, 2017) and others (Chen et al., 2008; Srivastava et al., 2010; Meng et al., 2013; Sharma et al., 2013; Dekker et al., 2016) have conducted several studies on NVP pharmacokinetics and toxicokinetics. These studies have clarified that NVP-induced toxicity is a biotransformation-driven process, associated with specific metabolic pathways and caused by specific reactive species. Taken together, these insights into

NVP lipid-friendly properties and biotransformation-related toxicity have prompted us to investigate the relations between NVP-based treatment, NVP biotransformation and NVP-induced modulation of HDL, particularly focusing on ApoA1 and PON-1 – two constituents of HDL proteome crucially involved in HDL functionality.

A brief summary of the relevant findings reported in the present dissertation, some considerations regarding the contributions of this work to the field of study, as well as the limitations of the research work and future studies needed, are presented below.

1. Summary of relevant findings

In the clinical study reported in Chapter 1, it was demonstrated that NVP treatment beneficially modulates HDL-cholesterol levels and, more importantly, HDL functionality. In fact, in both the prospective and the cross-sectional studies, NVP treatment was associated with higher levels of HDL-cholesterol and ApoA1, higher PON-1 activities, and lower levels of anti-HDL and anti-ApoA1 antibodies, reflecting an overall better functionality of HDL particles. We have also demonstrated that NVP-induced modulation of HDL quality is a drug-specific feature that was not observed when HIV-infected patients were treated with other antiretroviral schemes. Furthermore, sex was identified as a factor influencing NVP-induced modulation of HDL-cholesterol and ApoA1 levels, with women generally showing more beneficial changes upon NVP treatment. Interestingly, in the prospective clinical study (*cf.* Chapter 1), it was evident that NVP-induced modulation of different aspects of HDL functionality does not occur at the same time. The earliest event upon initiating NVP-based treatment was the decrease in the levels of anti-HDL antibodies, while the modulation of other study end-points was a later occurrence. This is an interesting observation considering the temporal changes in NVP metabolite profile, when patients increase NVP dose from the administration of 200 mg once daily to the steady-state 400 mg dose (Fan-Havard et al., 2013). In fact, there is an early predominance of 2-OH-NVP, while in the steady-state there is a decrease in the levels of 2-OH-NVP and a concomitant increase in the levels of 3-OH-NVP (Fan-Havard et al., 2013). NVP-induced CAR activation can lead to the induction of CYP2B6 (Faucette et al., 2006), the only CYP450 isoform involved in 3-OH-NVP formation (Erickson et al.,

1999), and also to the induction of several hepatic SULTs (Alnouti and Klaassen, 2008), therefore contributing to increased formation of 3-OH-NVP and concomitantly decreased levels of unconjugated 2-OH-NVP, through increased phase II conjugation of this metabolite. This explanation is coherent with our observation of steady-state NVP plasma levels strongly associated with 3-OH-NVP levels. This assumption is also consistent with a later modulatory effect on HDL, when there is enough accumulation of a NVP metabolite, as reported in Chapter 3, regarding the delayed modulation of ApoA1 the 3D hepatocyte model exposed to NVP and 2-OH-NVP. In fact, to complement the clinical studies, we performed *in vitro* studies employing three different models of hepatocytes, in order to isolate the individual contribution of NVP metabolites and to further examine the role of NVP biotransformation on the modulation of hepatic ApoA1 synthesis and PON-1 activities. These *in vitro* studies, reported in Chapter 2 and 3, demonstrated the usefulness of a more physiologically relevant hepatocyte system for studying the effects of drugs that undergo extensive phase I and phase II biotransformation, as is the case for NVP. We demonstrated that the boosting effect of NVP on hepatic ApoA1 seems to be dependent on 2-OH-NVP formation and subsequent biotransformation of this phase I metabolite. Regarding NVP-induced PON-1 modulation, the 12-OH-NVP formation seems to be the main factor mediating this effect. To the best of our knowledge, this is the first evidence on the significance of NVP biotransformation on the modulation of HDL functionality. Moreover, as far as we know this is the first time that a drug is associated with decreased levels of anti-HDL and anti-ApoA1 antibodies (Batuca, 2013).

These findings are particularly relevant considering the importance of PON-1 and ApoA1 as therapeutic targets. For instance, PON-1 has been implicated in the pathogenesis of several cardiovascular, metabolic, neurodegenerative and oncologic diseases (Table 3) and its pharmacological modulation has been already suggested for therapeutic purposes (Sena et al., 2013). Regarding ApoA1, the upregulation of this HDL-associated apolipoprotein is one of the most promising approaches to increase HDL-cholesterol levels and HDL function, and for the promotion of reverse cholesterol transport and long-term prevention of atherosclerosis (Gadkar et al., 2016). Moreover, and beyond the cardiovascular field, decreased levels of ApoA1 have been implicated as a causal

factor in ovarian cancer (Edelson, 2010; Su et al., 2010). For this reason, in Chapter 4 of this dissertation we have explored the effects of ApoA1 and an ApoA1 mimetic peptide on the malignant phenotype of ovarian cancer cells. The full-length ApoA1 and the 4F peptide were both able to decrease ovarian cancer cells viability. ApoA1 was able to decrease the invasiveness of cancer cells, while the ApoA1 mimetic peptide strongly supressed Akt signalling. In accordance with this effect on Akt pathway, the ApoA1 mimetic peptide was able to sensitise ovarian cancer cells to cisplatin, and this sensitisation effect was observed both *in vitro* and in the biologically relevant *in ovo* CAM model. These observations support the role of ApoA1 as a suppressor of ovarian tumorigenesis and as a chemosensitising agent in ovarian cancer. In addition, we have evaluated the levels of anti-HDL and anti-ApoA1 antibodies in women suffering from benign and malignant ovarian disease comparatively to healthy controls. However, no differences were observed on the levels of anti-HDL and anti-ApoA1 antibodies.

2. What are the contributions of the present work to the pharmacological field?

2-hydroxy-nevirapine as a template for the rational design of drugs aimed at increasing the systemic levels of apolipoprotein A1

The 2-OH-NVP metabolite was the compound identified as being responsible for the increase in hepatic synthesis of ApoA1 observed upon NVP exposure, in the 3D *in vitro* model of hepatocytes (*cf.* Chapter 3). Another interesting feature of 2-OH-NVP is its lower potential for toxicity. The formation of a reactive quinone-imine derived from oxidation of 2-OH-NVP is possible (Antunes et al., 2011), however this quinone-imine was only detected *in vitro*, after incubations of 2-OH-NVP with chemical oxidants and enzymes. The *in vivo* and clinical relevance of this reactive specie has yet to be confirmed, particularly because most of the 2-OH-NVP metabolite undergoes preferentially phase II sulfoconjugation and glucuronoconjugation (Pinheiro et al., 2017), instead of further oxidation.

Considering the effects of 2-OH-NVP in hepatic ApoA1 synthesis and its lower potential for toxicity, this NVP metabolite (or a 2-OH-NVP analogue with an even better safety

profile) could be useful for the rational design of drugs aimed at increasing the systemic levels of ApoA1. A drug with these ApoA1 booster properties would eventually benefit patients with dyslipidaemia characterized by low HDL-cholesterol levels and poor HDL functionality, and patients at increased risk of cardiovascular diseases and atherosclerosis. Moreover, the therapeutic applications of such compound could be expanded to gynaecological oncology, although further studies are needed to assess the benefits of an ApoA1 booster for oncologic patients.

A better understanding of the mechanisms involved in nevirapine-induced modulation of high density lipoprotein levels and functionality

The research work reported in Chapters 1, 2 and 3 of the current dissertation contributes to a better knowledge of how NVP biotransformation affects HDL modulation. Herein, we demonstrate that different biotransformation pathways might be involved in the modulation of different aspects of HDL functionality. The 2-OH-NVP metabolite seems to be more related with increases in hepatic synthesis of ApoA1, possibly upon phase II biotransformation of 2-OH-NVP (*cf.* Chapter 3), while the 12-OH-NVP metabolite seems to be more involved in the modulation of PON-1 activities (*cf.* Chapter 2).

A better knowledge of the mechanisms involved in the NVP modulatory effect on ApoA1 and PON-1 is essential and can pave the way for the development of new drugs targeting HDL functionality, as described above for 2-OH-NVP. Similarly, the 12-OH-NVP metabolite might be valuable for the rational design of an analogue, with a better toxicokinetic profile than 12-OH-NVP, targeting PON-1 activities. Such a molecule would possibly enrich the therapeutic arsenal for a wide range of pathological conditions in which oxidative stress plays a central role.

The identification of a better in vitro hepatocyte model for investigating the effects of small molecules subjected to extensive biotransformation

In Chapter 2 and 3, our experimental approach was based on the comparison between three different *in vitro* models of hepatocytes, which allowed the identification of the 3D hepatocyte system as the most suitable model for the study of ApoA1 and PON-1

modulation by NVP. In fact, the 3D hepatocyte model employed here mimics the *in vivo* cell organization, physiology and metabolic function (Miranda et al., 2009). Furthermore, the 3D culture of hepatocytes is able to effectively promote NVP biotransformation and bioactivation, generating the same metabolite profile observed *in vivo* (Pinheiro et al., 2017). Resorting to this methodological approach, we demonstrated that the selection of an adequate *in vitro* model is particularly relevant when investigating the pharmacological effects of compounds that suffer extensive phase I and phase II biotransformation, as is the case for NVP.

The identification of apolipoprotein A1 as an anti-tumorigenic protein and as a chemosensitising agent in ovarian cancer

In Chapter 4 we provided evidence showing that ApoA1 is able to modulate the malignant phenotype of ovarian cancer cells. By testing full-length human ApoA1 and a mimetic peptide, we demonstrated that these compounds can affect ovarian cancer cells viability, invasiveness, platinum sensitivity and oncogenic signalling through Akt. Importantly, the results regarding sensitisation to platinum were observed not only *in vitro* but also in the biologically relevant CAM model. These preliminary data support the hypothesis of ApoA1 as an anti-tumorigenic protein and as a platinum-sensitising agent, raising important questions regarding the application of ApoA1 boosters for treating ovarian malignancies. Theoretically, a therapy increasing the circulating levels of ApoA1 can have a positive impact on the ovarian cancer outcome by decreasing the ability of cancer cells to invade the extracellular matrix, which is an important initial step in the development of metastasis (Steeg, 2016), and by increasing the sensitivity of cancer cells to platinum-based chemotherapy. The sensitisation effect can be particularly relevant in the clinics since platinum resistance is a major challenge in ovarian cancer treatment (Gabra et al., 2008; Blagden and Gabra, 2009; Stronach et al., 2011; Foley et al., 2013; Cheraghchi-Bashi et al., 2015; Gungor et al., 2015).

3. Which are the limitations of the present work?

We have not tested the effect of minor NVP metabolites on PON-1 and ApoA1 in the *in vitro* hepatocyte models (*cf.* Chapter 2 and 3).

In Chapter 4, we have assessed the effect of ApoA1 and an ApoA1 mimetic peptide on the malignant phenotype of ovarian cancer cells. Likewise, other authors have also employed ApoA1 mimetic peptides for studying ApoA1 anti-tumorigenic properties (Su et al., 2010; Gkouskou et al., 2016). Despite the remarkable functional similarities between ApoA1 and its mimetic peptides (Datta et al., 2001; Xie et al., 2010; Mishra et al., 2013; Ying et al., 2013), these compounds can also present some functional differences (Datta et al., 2001; Van Lenten et al., 2008). One of these differences is related to LPA affinity (Van Lenten et al., 2008). In fact, while ApoA1 can effectively bind to LPA (Yeh et al., 2016), ApoA1 mimetic peptides present higher affinity towards this inflammatory phospholipid (Van Lenten et al., 2008). While not diminishing the relevance of the findings reported in Chapter 4, this difference in LPA affinity can be regarded as a limitation when using ApoA1 mimetic peptides as surrogates to investigate the anti-tumorigenic properties of ApoA1. Nevertheless, *in vivo* evidence (Su et al., 2010) supports that both overexpression of ApoA1 and treatment with ApoA1 mimetic peptides have a beneficial impact in terms of ovarian cancer development, leading to decreased tumour growth and prevention of metastasis formation, therefore arguing that the functional differences between ApoA1 and ApoA1 mimetic peptides are possibly less relevant in an *in vivo* system. Even so, in future studies, it would be important to evaluate the impact and the relevance of these differences.

Other limitations of the research work described in Chapter 4 are worthy of note. Firstly, the high embryo mortality in the CAM assay, which was caused by malfunctioning of the incubator, affecting temperature and humidity controls. Despite this methodological limitation, the *in ovo* findings confirmed the *in vitro* results regarding platinum sensitisation. Another limitation was the lack of clinical information for women included in the exploratory clinical study reported in Chapter 4.

4. What should be addressed in future studies?

The following topics highlight some aspects that were not addressed in the present research work and deserve consideration in future studies.

- Mechanistically, it would be worth investigate the effect of NVP metabolites in the activation of a panel of nuclear receptors that can mediate the upregulation of ApoA1 and PON-1; additionally, future studies might investigate if NVP and its metabolites are able to promote inhibition of the α-subunit of FNT and affect hepatic ApoA1 secretion, as investigated before for lopinavir and atazanavir (Coffinier et al., 2007);
- Investigate the effect of 2-OH-NVP and 2-OH-NVP sulfate as ApoA1 boosters for the treatment of dyslipidaemia and prevention of atherosclerosis *in vivo,* and evaluate its tolerability in animal models;
- Assess the effect of 12-OH-NVP analogues in terms of PON-1 modulation *in vitro,* in order to clarify if these compounds keep the ability to modulate PON-1; it would be important to fully characterize the toxicological profile of these analogues (*i.e.* potential to undergo bioactivation into reactive species, potential to form adducts with biomacromolecules and induce toxic reactions *in vivo*, etc.). It would be interesting to evaluate NVP derivatives as well. For instance, a NVP derivative blocked at carbon-12 position might retain PON-1 modulation ability and promote the preferential formation of 2-OH-NVP sulfate, allowing concomitant ApoA1 modulation;
- Regarding the effect of ApoA1 and ApoA1 mimetics in ovarian cancer, future studies must clarify the involvement of LPA sequestration, the modulation of oncogenic signalling in lipid rafts, and the contribution of other mechanisms to the anti-tumorigenic and sensitising effects mediated by these molecules;
- Finally, it would be worth investigating the potential of ApoA1 booster therapies, particularly NVP and 2-OH-NVP, in rodent models of ovarian cancer and compare the effect of these compounds with the administration of ApoA1 mimetic peptides, in terms of effectiveness and tolerability.

5. Conclusion

The re-profiling of commonly used drugs is an established strategy that allows existing molecules to be applied for new therapeutic indications (Langedijk et al., 2015), although the re-profiling of a metabolite, derivative or analogue of a well-known drug into a new active pharmaceutical ingredient is less common (Mazerbourg et al., 2016). Drug re-profiling can be a rich source of therapeutic solutions for several diseases, allowing the emergence of new treatments at a lower cost comparatively to the traditional drug development process (Dilly and Morris, 2017). As discussed along this dissertation, NVP has been used in the clinical practice for more than two decades (Bowersox, 1996) and its pharmacological and toxicological profile has been extensively studied (Erickson et al., 1999; Lamson et al., 1999; Riska et al., 1999a, 1999b; Antunes et al., 2008, 2010a, 2010b, 2011; Chen et al., 2008; Wen et al., 2009; Popovic et al., 2010; Srivastava et al., 2010; Yuan et al., 2011; Caixas et al., 2012; Meng et al., 2013; Sharma et al., 2013; Kranendonk et al., 2014; Marinho et al., 2014a, 2014b, 2014c; Pinheiro et al., 2015, 2017; Dekker et al., 2016) as well as its positive impact on the lipid profile (van der Valk et al., 2001; Ruiz et al., 2001; Clotet et al., 2003; van Leth et al., 2004; Sankatsing et al., 2007; Franssen et al., 2009; Podzamczer et al., 2011, 2014; Strehlau et al., 2012; Arpadi et al., 2013; Marinho et al., 2014a, 2016). Here, we have contributed to a better understanding of the role of biotransformation in NVP-induced modulation of HDL particle functionality, particularly focusing on ApoA1 and PON-1. This biotransformation-oriented approach is really innovative considering that drug biotransformation has been always neglected as a determinant factor for NVP lipid-friendly properties, even in more mechanistic studies resorting to sophisticated methodologies as the research reported by Franssen and collaborators (2009). Moreover, we have also provided new insights into the potential of ApoA1 in ovarian cancer therapy, either alone or in combination with chemotherapeutic drugs, although further studies must be conducted to assess the usefulness of HDL/ApoA1 booster therapies in gynaecological oncology. In conclusion, the re-profiling of NVP as an HDL modulator seems a reasonable and suitable strategy for the treatment of diseases associated with poor HDL functionality, from cardiovascular diseases to cancer, or even for the prevention of such pathologies. Hopefully, the research reported in this

dissertation will contribute to the development of drugs that can bring real benefits to several patients.

REFERENCES

Aberg, J.A., 2012. Aging, inflammation, and HIV infection. Top. Antivir. Med. 20, 101–105.
Agarwal, A., Sharma, R., Durairajanayagam, D., Ayaz, A., Cui, Z., Willard, B., Gopalan, B., Sabanegh, E., 2015. Major protein alterations in spermatozoa from infertile men with unilateral varicocele. Reprod. Biol. Endocrinol. 13. https://doi.org/10.1186/s12958-015-0007-2
Allan, G.F., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1992. Ligand-dependent conformational changes in the progesterone receptor are necessary for events that follow DNA binding. Proc. Natl. Acad. Sci. U. S. A. 89, 11750–11754.
Alnouti, Y., Klaassen, C.D., 2011. Mechanisms of gender-specific regulation of mouse sulfotransferases (Sults). Xenobiotica 41, 187–197. https://doi.org/10.3109/00498254.2010.535923
Alnouti, Y., Klaassen, C.D., 2008. Regulation of Sulfotransferase Enzymes by Prototypical Microsomal Enzyme Inducers in Mice. J. Pharmacol. Exp. Ther. 324, 612–621. https://doi.org/10.1124/jpet.107.129650
Alnouti, Y., Klaassen, C.D., 2006. Tissue distribution and ontogeny of sulfotransferase enzymes in mice. Toxicol. Sci. Off. J. Soc. Toxicol. 93, 242–255. https://doi.org/10.1093/toxsci/kfl050
Altomare, D.A., Testa, J.R., 2005. Perturbations of the AKT signaling pathway in human cancer. Oncogene 24, 7455–7464. https://doi.org/10.1038/sj.onc.1209085
Antinori, A., Baldini, F., Girardi, E., Cingolani, A., Zaccarelli, M., Di Giambenedetto, S., Barracchini, A., De Longis, P., Murri, R., Tozzi, V., Ammassari, A., Rizzo, M.G., Ippolito, G., De Luca, A., 2001. Female sex and the use of anti-allergic agents increase the risk of developing cutaneous rash associated with nevirapine therapy. AIDS Lond. Engl. 15, 1579–1581.
Antony, J., Tan, T.Z., Kelly, Z., Low, J., Choolani, M., Recchi, C., Gabra, H., Thiery, J.P., Huang, R.Y.-J., 2016. The GAS6-AXL signaling network is a mesenchymal (Mes) molecular subtype-specific therapeutic target for ovarian cancer. Sci. Signal. 9, ra97-ra97. https://doi.org/10.1126/scisignal.aaf8175
Antunes, A.M.M., Duarte, M.P., Santos, P.P., Gamboa da Costa, G., Heinze, T.M., Beland, F.A., Marques, M.M., 2008. Synthesis and Characterization of DNA Adducts from the HIV Reverse Transcriptase Inhibitor Nevirapine. Chem. Res. Toxicol. 21, 1443–1456. https://doi.org/10.1021/tx8000972
Antunes, A.M.M., Godinho, A.L.A., Martins, I.L., Justino, G.C., Beland, F.A., Marques, M.M., 2010a. Amino Acid Adduct Formation by the Nevirapine Metabolite, 12-Hydroxynevirapine—A Possible Factor in Nevirapine Toxicity. Chem. Res. Toxicol. 23, 888–899. https://doi.org/10.1021/tx900443z
Antunes, A.M.M., Godinho, A.L.A., Martins, I.L., Oliveira, M.C., Gomes, R.A., Coelho, A.V., Beland, F.A., Marques, M.M., 2010b. Protein Adducts As Prospective Biomarkers of Nevirapine Toxicity. Chem. Res. Toxicol. 23, 1714–1725. https://doi.org/10.1021/tx100186t
Antunes, A.M.M., Novais, D.A., Ferreira da Silva, J.L., Santos, P.P., Conceição Oliveira, M., Beland, F.A., Matilde Marques, M., 2011. Synthesis and oxidation of 2-hydroxynevirapine, a metabolite of the HIV reverse transcriptase inhibitor nevirapine. Org. Biomol. Chem. 9, 7822. https://doi.org/10.1039/c1ob06052j
Arab-Alameddine, M., Décosterd, L.A., Buclin, T., Telenti, A., Csajka, C., 2011. Antiretroviral drug toxicity in relation to pharmacokinetics, metabolic profile and pharmacogenetics. Expert Opin. Drug Metab. Toxicol. 7, 609–622. https://doi.org/10.1517/17425255.2011.562891
Ariel, M., Cedar, H., McCarrey, J., 1994. Developmental changes in methylation of spermatogenesis–specific genes include reprogramming in the epididymis. Nat. Genet. 7, 59–63. https://doi.org/10.1038/ng0594-59

Arii, K., Suehiro, T., Ota, K., Ikeda, Y., Kumon, Y., Osaki, F., Inoue, M., Inada, S., Ogami, N., Takata, H., Hashimoto, K., Terada, Y., 2009. Pitavastatin induces PON1 expression through p44/42 mitogen-activated protein kinase signaling cascade in Huh7 cells. Atherosclerosis 202, 439–445. https://doi.org/10.1016/j.atherosclerosis.2008.05.013

Arpadi, S., Shiau, S., Strehlau, R., Martens, L., Patel, F., Coovadia, A., Abrams, E.J., Kuhn, L., 2013. Metabolic abnormalities and body composition of HIV-infected children on Lopinavir or Nevirapine-based antiretroviral therapy. Arch. Dis. Child. 98, 258–264. https://doi.org/10.1136/archdischild-2012-302633

Arts, E.J., Hazuda, D.J., 2012. HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb. Perspect. Med. 2, a007161–a007161. https://doi.org/10.1101/cshperspect.a007161

Aviram, M., Hardak, E., Vaya, J., Mahmood, S., Milo, S., Hoffman, A., Billicke, S., Draganov, D., Rosenblat, M., 2000. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. Circulation 101, 2510–2517.

Ayala, A., Muñoz, M.F., Argüelles, S., 2014. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid. Med. Cell. Longev. 2014, 1–31. https://doi.org/10.1155/2014/360438

Aydin, O., Yacinkaya, S., Eren, E., Ergin, M., Eroglu, M., Yilmaz, N., 2013. Diminished arylesterase enzyme activity and total thiol levels in bladder cancer patients. Clin. Lab. 59, 1231–1237.

Bacchetti, T., Vignini, A., Giulietti, A., Nanetti, L., Provinciali, L., Luzzi, S., Mazzanti, L., Ferretti, G., 2015. Higher Levels of Oxidized Low Density Lipoproteins in Alzheimer's Disease Patients: Roles for Platelet Activating Factor Acetyl Hydrolase and Paraoxonase-1. J. Alzheimers Dis. JAD 46, 179–186. https://doi.org/10.3233/JAD-143096

Bachmann, K., Patel, H., Batayneh, Z., Slama, J., White, D., Posey, J., Ekins, S., Gold, D., Sambucetti, L., 2004. PXR and the regulation of apoA1 and HDL-cholesterol in rodents. Pharmacol. Res. 50, 237–246. https://doi.org/10.1016/j.phrs.2004.03.005

Balci, H., Genc, H., Papila, C., Can, G., Papila, B., Yanardag, H., Uzun, H., 2012. Serum Lipid Hydroperoxide Levels and Paraoxonase Activity in Patients With Lung, Breast, and Colorectal Cancer: Paraoxonase and Cancer. J. Clin. Lab. Anal. 26, 155–160. https://doi.org/10.1002/jcla.21503

Ballantyne, C.M., 2003. Effect of Ezetimibe Coadministered With Atorvastatin in 628 Patients With Primary Hypercholesterolemia: A Prospective, Randomized, Double-Blind Trial. Circulation 107, 2409–2415. https://doi.org/10.1161/01.CIR.0000068312.21969.C8

Barnett, A.C., Tsvetanov, S., Gamage, N., Martin, J.L., Duggleby, R.G., McManus, M.E., 2004. Active Site Mutations and Substrate Inhibition in Human Sulfotransferase 1A1 and 1A3. J. Biol. Chem. 279, 18799–18805. https://doi.org/10.1074/jbc.M312253200

Barré-Sinoussi, F., Ross, A.L., Delfraissy, J.-F., 2013. Past, present and future: 30 years of HIV research. Nat. Rev. Microbiol. 11, 877–883. https://doi.org/10.1038/nrmicro3132

Barter, P.J., Caulfield, M., Eriksson, M., Grundy, S.M., Kastelein, J.J.P., Komajda, M., Lopez-Sendon, J., Mosca, L., Tardif, J.-C., Waters, D.D., Shear, C.L., Revkin, J.H., Buhr, K.A., Fisher, M.R., Tall, A.R., Brewer, B., 2007. Effects of Torcetrapib in Patients at High Risk for Coronary Events. N. Engl. J. Med. 357, 2109–2122. https://doi.org/10.1056/NEJMoa0706628

Bast, R.C., Hennessy, B., Mills, G.B., 2009. The biology of ovarian cancer: new opportunities for translation. Nat. Rev. Cancer 9, 415–428. https://doi.org/10.1038/nrc2644

Batuca, J.R., 2013. Humoral response towards high density lipoprotein : a new mechanism for atherogenesis. (Doctoral thesis). Universidade Nova de Lisboa., Lisbon.

Batuca, J.R., Amaral, M.C., Favas, C., Paula, F.S., Ames, P.R.J., Papoila, A.L., Delgado Alves, J., 2016. Extended-release niacin increases anti-apolipoprotein A-I antibodies that block the antioxidant effect of high-density lipoprotein-cholesterol: the EXPLORE clinical

trial: ERN, anti-ApoA1 antibodies and their effect on HDL-C. Br. J. Clin. Pharmacol. https://doi.org/10.1111/bcp.13198

Batuca, J.R., Ames, P.R.J., Amaral, M., Favas, C., Isenberg, D.A., Delgado Alves, J., 2008. Anti-atherogenic and anti-inflammatory properties of high-density lipoprotein are affected by specific antibodies in systemic lupus erythematosus. Rheumatology 48, 26–31. https://doi.org/10.1093/rheumatology/ken397

Batuca, J.R., Ames, P.R.J., Isenberg, D.A., Alves, J.D., 2007. Antibodies toward high-density lipoprotein components inhibit paraoxonase activity in patients with systemic lupus erythematosus. Ann. N. Y. Acad. Sci. 1108, 137–146.

Bauvois, B., 2012. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: Outside-in signaling and relationship to tumor progression. Biochim. Biophys. Acta BBA - Rev. Cancer 1825, 29–36. https://doi.org/10.1016/j.bbcan.2011.10.001

Becker, S., von Otte, S., Robenek, H., Diedrich, K., Nofer, J.-R., 2011. Follicular Fluid High-Density Lipoprotein-Associated Sphingosine 1-Phosphate (S1P) Promotes Human Granulosa Lutein Cell Migration via S1P Receptor Type 3 and Small G-Protein RAC11. Biol. Reprod. 84, 604–612. https://doi.org/10.1095/biolreprod.110.084152

Beloribi-Djefaflia, S., Vasseur, S., Guillaumond, F., 2016. Lipid metabolic reprogramming in cancer cells. Oncogenesis 5, e189. https://doi.org/10.1038/oncsis.2015.49

Bera, E., Mia, R., 2012. Safety of nevirapine in HIV-infected pregnant women initiating antiretroviral therapy at higher CD4 counts: A systematic review and meta-analysis. S. Afr. Med. J. 102, 855. https://doi.org/10.7196/SAMJ.5700

Bersoff-Matcha, S.J., Miller, W.C., Aberg, J.A., van der Horst, C., Hamrick, H.J., Powderly, W.G., Mundy, L.M., 2001. Sex Differences in Nevirapine Rash. Clin. Infect. Dis. 32, 124–129. https://doi.org/10.1086/317536

Bian, H.S., Ngo, S.Y.Y., Tan, W., Wong, C.H., Boelsterli, U.A., Tan, T.M.C., 2007. Induction of human sulfotransferase 1A3 (SULT1A3) by glucocorticoids. Life Sci. 81, 1659–1667. https://doi.org/10.1016/j.lfs.2007.09.029

Bigley, A.N., Raushel, F.M., 2013. Catalytic mechanisms for phosphotriesterases. Biochim. Biophys. Acta BBA - Proteins Proteomics 1834, 443–453. https://doi.org/10.1016/j.bbapap.2012.04.004

Bisoendial, R., Tabet, F., Tak, P.P., Petrides, F., Cuesta Torres, L.F., Hou, L., Cook, A., Barter, P.J., Weninger, W., Rye, K.-A., 2015. Apolipoprotein A-I Limits the Negative Effect of Tumor Necrosis Factor on LymphangiogenesisSignificance. Arterioscler. Thromb. Vasc. Biol. 35, 2443–2450. https://doi.org/10.1161/ATVBAHA.115.305777

Blagden, S., Gabra, H., 2009. Promising molecular targets in ovarian cancer: Curr. Opin. Oncol. 21, 412–419. https://doi.org/10.1097/CCO.0b013e32832eab1f

Bloedon, L.T., Dunbar, R., Duffy, D., Pinell-Salles, P., Norris, R., DeGroot, B.J., Movva, R., Navab, M., Fogelman, A.M., Rader, D.J., 2008. Safety, pharmacokinetics, and pharmacodynamics of oral apoA-I mimetic peptide D-4F in high-risk cardiovascular patients. J. Lipid Res. 49, 1344–1352. https://doi.org/10.1194/jlr.P800003-JLR200

Bocedi, A., Notaril, S., Narciso, P., Bolli, A., Fasano, M., Ascenzi, P., 2004. Binding of Anti-HIV Drugs to Human Serum Albumin. IUBMB Life Int. Union Biochem. Mol. Biol. Life 56, 609–614. https://doi.org/10.1080/15216540400016286

Bogan, R.L., DeBarber, A.E., Hennebold, J.D., 2012. Liver X Receptor Modulation of Gene Expression Leading to Proluteolytic Effects in Primate Luteal Cells1. Biol. Reprod. 86. https://doi.org/10.1095/biolreprod.111.096347

Bogan, R.L., Hennebold, J.D., 2010. The reverse cholesterol transport system as a potential mediator of luteolysis in the primate corpus luteum. Reproduction 139, 163–176. https://doi.org/10.1530/REP-09-0005

Bouman, H.J., Schömig, E., van Werkum, J.W., Velder, J., Hackeng, C.M., Hirschhäuser, C., Waldmann, C., Schmalz, H.-G., ten Berg, J.M., Taubert, D., 2011. Paraoxonase-1 is a

major determinant of clopidogrel efficacy. Nat. Med. 17, 110–116. https://doi.org/10.1038/nm.2281

Bowersox, J., 1996. Nevirapine approved by FDA. Food and Drug Administration. NIAID AIDS Agenda. 10.

Brasseur, K., Gévry, N., Asselin, E., 2017. Chemoresistance and targeted therapies in ovarian and endometrial cancers. Oncotarget. https://doi.org/10.18632/oncotarget.14021

Bright, A.S., Herrera-Garcia, G., E. Moscovitz, J., You, D., L. Guo, G., M. Aleksunes, L., 2016. Regulation of Drug Disposition Gene Expression in Pregnant Mice with Car Receptor Activation. Nucl. Recept. Res. 3. https://doi.org/10.11131/2016/101193

Bryant, A.K., Fazeli, P.L., Letendre, S.L., Ellis, R.J., Potter, M., Burdo, T.H., Singh, K.K., Jeste, D.V., Grant, I., Moore, D.J., 2016. Complement Component 3 Is Associated with Metabolic Comorbidities in Older HIV-Positive Adults. AIDS Res. Hum. Retroviruses 32, 271–278. https://doi.org/10.1089/aid.2015.0179

Bulbuller, N., Eren, E., Ellidag, H.Y., Oner, O.Z., Sezer, C., Aydin, O., Yılmaz, N., 2013. Diagnostic value of thiols, paraoxonase 1, arylesterase and oxidative balance in colorectal cancer in human. Neoplasma 60, 419–424. https://doi.org/10.4149/neo_2013_054

Byakika-Kibwika, P., Lamorde, M., Mayito, J., Nabukeera, L., Namakula, R., Mayanja-Kizza, H., Katabira, E., Ntale, M., Pakker, N., Ryan, M., Hanpithakpong, W., Tarning, J., Lindegardh, N., de Vries, P.J., Khoo, S., Back, D., Merry, C., 2012. Significant pharmacokinetic interactions between artemether/lumefantrine and efavirenz or nevirapine in HIV-infected Ugandan adults. J. Antimicrob. Chemother. 67, 2213–2221. https://doi.org/10.1093/jac/dks207

Byrne, A.T., Ross, L., Holash, J., Nakanishi, M., Hu, L., Hofmann, J.I., Yancopoulos, G.D., Jaffe, R.B., 2003. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 9, 5721–5728.

Cai, H., Xu, Y., 2013. The role of LPA and YAP signaling in long-term migration of human ovarian cancer cells. Cell Commun. Signal. 11, 31. https://doi.org/10.1186/1478-811X-11-31

Caixas, U., Antunes, A.M.M., Marinho, A.T., Godinho, A.L.A., Grilo, N.M., Marques, M.M., Oliveira, M.C., Branco, T., Monteiro, E.C., Pereira, S.A., 2012. Evidence for nevirapine bioactivation in man: Searching for the first step in the mechanism of nevirapine toxicity. Toxicology 301, 33–39. https://doi.org/10.1016/j.tox.2012.06.013

Calcagno, A., Di Perri, G., Bonora, S., 2014. Pharmacokinetics and Pharmacodynamics of Antiretrovirals in the Central Nervous System. Clin. Pharmacokinet. 53, 891–906. https://doi.org/10.1007/s40262-014-0171-0

Camont, L., Chapman, M.J., Kontush, A., 2011. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. Trends Mol. Med. 17, 594–603. https://doi.org/10.1016/j.molmed.2011.05.013

Camont, L., Lhomme, M., Rached, F., Le Goff, W., Negre-Salvayre, A., Salvayre, R., Calzada, C., Lagarde, M., Chapman, M.J., Kontush, A., 2013. Small, Dense High-Density Lipoprotein-3 Particles Are Enriched in Negatively Charged Phospholipids: Relevance to Cellular Cholesterol Efflux, Antioxidative, Antithrombotic, Anti-Inflammatory, and Antiapoptotic Functionalities. Arterioscler. Thromb. Vasc. Biol. 33, 2715–2723. https://doi.org/10.1161/ATVBAHA.113.301468

Camps, J., Iftimie, S., García-Heredia, A., Castro, A., Joven, J., 2017. Paraoxonases and infectious diseases. Clin. Biochem. https://doi.org/10.1016/j.clinbiochem.2017.04.016

Camps, J., Marsillach, J., Joven, J., 2009. Measurement of serum paraoxonase-1 activity in the evaluation of liver function. World J. Gastroenterol. 15, 1929. https://doi.org/10.3748/wjg.15.1929

Camps, J., Pujol, I., Ballester, F., Joven, J., Simo, J.M., 2011. Paraoxonases as Potential Antibiofilm Agents: Their Relationship with Quorum-Sensing Signals in Gram-Negative

Bacteria. Antimicrob. Agents Chemother. 55, 1325–1331. https://doi.org/10.1128/AAC.01502-10

Camuzcuoglu, H., Arioz, D.T., Toy, H., Kurt, S., Celik, H., Erel, O., 2009. Serum paraoxonase and arylesterase activities in patients with epithelial ovarian cancer. Gynecol. Oncol. 112, 481–485. https://doi.org/10.1016/j.ygyno.2008.10.031

Carlini, E.J., Raftogianis, R.B., Wood, T.C., Jin, F., Zheng, W., Rebbeck, T.R., Weinshilboum, R.M., 2001. Sulfation pharmacogenetics: SULT1A1 and SULT1A2 allele frequencies in Caucasian, Chinese and African-American subjects. Pharmacogenetics 11, 57–68.

Carr, D.F., Chaponda, M., Jorgensen, A.L., Castro, E.C., van Oosterhout, J.J., Khoo, S.H., Lalloo, D.G., Heyderman, R.S., Alfirevic, A., Pirmohamed, M., 2013. Association of Human Leukocyte Antigen Alleles and Nevirapine Hypersensitivity in a Malawian HIV-Infected Population. Clin. Infect. Dis. 56, 1330–1339. https://doi.org/10.1093/cid/cit021

Castillo-Juarez, I., López-Jácome, L.E., Soberón-Chávez, G., Tomás, M., Lee, J., Castañeda-Tamez, P., Hernández-Bárragan, I.Á., Cruz-Muñiz, M.Y., Maeda, T., Wood, T.K., García-Contreras, R., 2017. Exploiting Quorum Sensing Inhibition for the Control of Pseudomonas Aeruginosa and Acinetobacter Baumannii Biofilms. Curr. Top. Med. Chem.

Catapano, A., Brady, W.E., King, T.R., Palmisano, J., 2005. Lipid altering-efficacy of ezetimibe co-administered with simvastatin compared with rosuvastatin: a meta-analysis of pooled data from 14 clinical trials. Curr. Med. Res. Opin. 21, 1123–1130. https://doi.org/10.1185/030079905X50642

Cattelan, A.M., Erne, E., Salatino, A., Trevenzoli, M., Carretta, G., Meneghetti, F., Cadrobbi, P., 1999. Severe Hepatic Failure Related to Nevirapine Treatment. Clin. Infect. Dis. 29, 455–456. https://doi.org/10.1086/520242

Çebi, A., Akgun, E., Esen, R., Demir, H., Çifci, A., 2015. The activities of serum paraoxonase and arylesterase and lipid profile in acute myeloid leukemia: preliminary results. Eur. Rev. Med. Pharmacol. Sci. 19, 4590–4594.

Chang, T.K.H., Waxman, D.J., 2005. Enzymatic Analysis of cDNA-Expressed Human CYP1A1, CYP1A2, and CYP1B1 With 7-Ethoxyresorufin as Substrate, in: Cytochrome P450 Protocols. Humana Press, New Jersey, pp. 85–90. https://doi.org/10.1385/1-59259-998-2:85

Chantarangsu, S., Mushiroda, T., Mahasirimongkol, S., Kiertiburanakul, S., Sungkanuparph, S., Manosuthi, W., Tantisiriwat, W., Charoenyingwattana, A., Sura, T., Chantratita, W., Nakamura, Y., 2009. HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients: Pharmacogenet. Genomics 19, 139–146. https://doi.org/10.1097/FPC.0b013e32831d0faf

Chen, G., Zhang, D., Jing, N., Yin, S., Falany, C.N., Radominska-Pandya, A., 2003. Human gastrointestinal sulfotransferases: identification and distribution. Toxicol. Appl. Pharmacol. 187, 186–197.

Chen, J., Mannargudi, B.M., Xu, L., Uetrecht, J., 2008. Demonstration of the Metabolic Pathway Responsible for Nevirapine-Induced Skin Rash. Chem. Res. Toxicol. 21, 1862–1870. https://doi.org/10.1021/tx800177k

Chen, J., Zhu, L., Li, X., Zheng, H., Yan, T., Xie, C., Zeng, S., Yu, J., Jiang, H., Lu, L., Qi, X., Wang, Y., Hu, M., Liu, Z., 2017. High Throughput and Reliable Isotope Label-free approach for Profiling 24 Metabolic Enzymes in FVB Mice and Gender Differences. Drug Metab. Dispos. dmd.116.074682. https://doi.org/10.1124/dmd.116.074682

Cheng, C.-C., Hsueh, C.-M., Liang, K.-W., Ting, C.-T., Wen, C.-L., Hsu, S.-L., 2011. Role of JNK and c-Jun signaling pathway in regulation of human serum paraoxonase 1 gene transcription by berberine in human HepG2 cells. Eur. J. Pharmacol. 650, 519–525. https://doi.org/10.1016/j.ejphar.2010.10.051

Cheng, T., Dai, X., Zhou, D.-L., Lv, Y., Miao, L.-Y., 2015. Correlation of apolipoprotein A-I kinetics with survival and response to first-line platinum-based chemotherapy in advanced

non-small cell lung cancer. Med. Oncol. 32. https://doi.org/10.1007/s12032-014-0407-8

Cheng, X., Klaassen, C.D., 2012. Hormonal and Chemical Regulation of Paraoxonases in Mice. J. Pharmacol. Exp. Ther. 342, 688–695. https://doi.org/10.1124/jpet.112.194803

Cheraghchi-Bashi, A., Parker, C.A., Curry, E., Salazar, J.-F., Gungor, H., Saleem, A., Cunnea, P., Rama, N., Salinas, C., Mills, G.B., Morris, S.R., Kumar, R., Gabra, H., Stronach, E.A., 2015. A putative biomarker signature for clinically effective AKT inhibition: correlation of *in vitro*, *in vivo* and clinical data identifies the importance of modulation of the mTORC1 pathway. Oncotarget 6, 41736–41749. https://doi.org/10.18632/oncotarget.6153

Cheung, M., Testa, J.R., 2013. Diverse mechanisms of AKT pathway activation in human malignancy. Curr. Cancer Drug Targets 13, 234–244.

Chistiakov, D.A., Melnichenko, A.A., Orekhov, A.N., Bobryshev, Y.V., 2017. Paraoxonase and atherosclerosis-related cardiovascular diseases. Biochimie 132, 19–27. https://doi.org/10.1016/j.biochi.2016.10.010

Chistiakov, D.A., Orekhov, A.N., Bobryshev, Y.V., 2016. ApoA1 and ApoA1-specific self-antibodies in cardiovascular disease. Lab. Invest. 96, 708–718. https://doi.org/10.1038/labinvest.2016.56

Choi, S.-Y., Koh, K.H., Jeong, H., 2013. Isoform-Specific Regulation of Cytochromes P450 Expression by Estradiol and Progesterone. Drug Metab. Dispos. 41, 263–269. https://doi.org/10.1124/dmd.112.046276

Chong, P.-K., Lee, H., Zhou, J., Liu, S.-C., Loh, M.C.S., So, J.B.Y., Lim, K.H., Yeoh, K.-G., Lim, Y.-P., 2010. Reduced plasma APOA1 level is associated with Gastric Tumor Growth in MKN45 mouse xenograft model. J. Proteomics 73, 1632–1640. https://doi.org/10.1016/j.jprot.2010.04.005

Chou, C.-H., Wei, L.-H., Kuo, M.-L., Huang, Y.-J., Lai, K.-P., Chen, C.-A., Hsieh, C.-Y., 2004. Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF- B pathway by lysophosphatidic acid, an ovarian cancer-activating factor. Carcinogenesis 26. https://doi.org/10.1093/carcin/bgh301

Chow, W.A., Jiang, C., Guan, M., 2009. Anti-HIV drugs for cancer therapeutics: back to the future? Lancet Oncol. 10, 61–71. https://doi.org/10.1016/S1470-2045(08)70334-6

Christianson, M.S., Yates, M., 2012. Scavenger receptor class B type 1 gene polymorphisms and female fertility: Curr. Opin. Endocrinol. Diabetes Obes. 19, 115–120. https://doi.org/10.1097/MED.0b013e3283505771

Clarke, C.H., Yip, C., Badgwell, D., Fung, E.T., Coombes, K.R., Zhang, Z., Lu, K.H., Bast, R.C., 2011. Proteomic biomarkers apolipoprotein A1, truncated transthyretin and connective tissue activating protein III enhance the sensitivity of CA125 for detecting early stage epithelial ovarian cancer. Gynecol. Oncol. 122, 548–553. https://doi.org/10.1016/j.ygyno.2011.06.002

Clotet, B., van der Valk, M., Negredo, E., Reiss, P., 2003. Impact of nevirapine on lipid metabolism. J. Acquir. Immune Defic. Syndr. 1999 34 Suppl 1, S79-84.

Coburn, S.B., Bray, F., Sherman, M.E., Trabert, B., 2017. International patterns and trends in ovarian cancer incidence, overall and by histologic subtype: Ovarian cancer trends. Int. J. Cancer 140, 2451–2460. https://doi.org/10.1002/ijc.30676

Coffinier, C., Hudon, S.E., Farber, E.A., Chang, S.Y., Hrycyna, C.A., Young, S.G., Fong, L.G., 2007. HIV protease inhibitors block the zinc metalloproteinase ZMPSTE24 and lead to an accumulation of prelamin A in cells. Proc. Natl. Acad. Sci. 104, 13432–13437. https://doi.org/10.1073/pnas.0704212104

Costa, L.G., Vitalone, A., Cole, T.B., Furlong, C.E., 2005. Modulation of paraoxonase (PON1) activity. Biochem. Pharmacol. 69, 541–550. https://doi.org/10.1016/j.bcp.2004.08.027

Coughtrie, M.W.H., 2016. Function and organization of the human cytosolic sulfotransferase (SULT) family. Chem. Biol. Interact. 259, 2–7. https://doi.org/10.1016/j.cbi.2016.05.005

Dahabreh, D.F., Medh, J.D., 2012. Activation of peroxisome proliferator activated receptor-gamma results in an atheroprotective apolipoprotein profile in HepG2 cells. Adv. Biol. Chem. 02, 218–225. https://doi.org/10.4236/abc.2012.23026

Daminelli, E.N., Spada, C., Treitinger, A., Oliveira, T.V., Latrilha, M. da C., Maranhão, R.C., 2008. Alterations in lipid transfer to High-Density Lipoprotein (HDL) and activity of paraoxonase-1 in HIV+ patients. Rev. Inst. Med. Trop. São Paulo 50, 223–227. https://doi.org/10.1590/S0036-46652008000400007

Datta, G., Chaddha, M., Hama, S., Navab, M., Fogelman, A.M., Garber, D.W., Mishra, V.K., Epand, R.M., Epand, R.F., Lund-Katz, S., Phillips, M.C., Segrest, J.P., Anantharamaiah, G.M., 2001. Effects of increasing hydrophobicity on the physical-chemical and biological properties of a class A amphipathic helical peptide. J. Lipid Res. 42, 1096–1104.

Davidson, M.H., Rosenson, R.S., Maki, K.C., Nicholls, S.J., Ballantyne, C.M., Mazzone, T., Carlson, D.M., Williams, L.A., Kelly, M.T., Camp, H.S., Lele, A., Stolzenbach, J.C., 2014. Effects of Fenofibric Acid on Carotid Intima-Media Thickness in Patients With Mixed Dyslipidemia on Atorvastatin Therapy: Randomized, Placebo-Controlled Study (FIRST). Arterioscler. Thromb. Vasc. Biol. 34, 1298–1306. https://doi.org/10.1161/ATVBAHA.113.302926

Davies, B., Steele, I.A., Edmondson, R.J., Zwolinski, S.A., Saretzki, G., von Zglinicki, T., O'Hare, M.J., 2003. Immortalisation of human ovarian surface epithelium with telomerase and temperature-senstitive SV40 large T antigen. Exp. Cell Res. 288, 390–402. https://doi.org/10.1016/S0014-4827(03)00218-0

Davis, K.A., Crow, J.A., Chambers, H.W., Meek, E.C., Chambers, J.E., 2009. Racial Differences in Paraoxonase-1 (PON1): A Factor in the Health of Southerners? Environ. Health Perspect. 117, 1226–1231. https://doi.org/10.1289/ehp.0900569

de Melo, A.C., Paulino, E., Garces, Á.H.I., 2017. A Review of mTOR Pathway Inhibitors in Gynecologic Cancer. Oxid. Med. Cell. Longev. 2017, 1–8. https://doi.org/10.1155/2017/4809751

Deakin, S., Guernier, S., James, R.W., 2007. Pharmacogenetic interaction between paraoxonase-1 gene promoter polymorphism C-107T and statin: Pharmacogenet. Genomics 17, 451–457. https://doi.org/10.1097/FPC.0b013e3280925716

Deakin, S., Leviev, I., Gomaraschi, M., Calabresi, L., Franceschini, G., James, R.W., 2002. Enzymatically Active Paraoxonase-1 Is Located at the External Membrane of Producing Cells and Released by a High Affinity, Saturable, Desorption Mechanism. J. Biol. Chem. 277, 4301–4308. https://doi.org/10.1074/jbc.M107440200

Deakin, S., Moren, X., James, R.W., 2005. Very low density lipoproteins provide a vector for secretion of paraoxonase-1 from cells. Atherosclerosis 179, 17–25. https://doi.org/10.1016/j.atherosclerosis.2004.08.039

Deakin, S.P., Bioletto, S., Bochaton-Piallat, M.-L., James, R.W., 2011. HDL-associated paraoxonase-1 can redistribute to cell membranes and influence sensitivity to oxidative stress. Free Radic. Biol. Med. 50, 102–109. https://doi.org/10.1016/j.freeradbiomed.2010.09.002

Dekker, S.J., Zhang, Y., Vos, J.C., Vermeulen, N.P.E., Commandeur, J.N.M., 2016. Different Reactive Metabolites of Nevirapine Require Distinct Glutathione *S* -Transferase Isoforms for Bioinactivation. Chem. Res. Toxicol. 29, 2136–2144. https://doi.org/10.1021/acs.chemrestox.6b00250

Delerive, P., Galardi, C.M., Bisi, J.E., Nicodeme, E., Goodwin, B., 2004. Identification of Liver Receptor Homolog-1 as a Novel Regulator of Apolipoprotein AI Gene Transcription. Mol. Endocrinol. 18, 2378–2387. https://doi.org/10.1210/me.2004-0132

Dias, C., Marinho, A., Morello, J., Almeida, G., Caixas, U., Soto, K., Monteiro, E., Pereira, S., 2014a. Monitoring of the lactonase activity of paraoxonase-1 enzyme in HIV-1-infection. J. Int. AIDS Soc. 17. https://doi.org/10.7448/IAS.17.4.19682

Dias, C.G., 2013. HDL in HIV-1 infection: a quality perspective through paraoxonase-1 activities. (Dissertação de Mestrado). Universidade Nova de Lisboa., Faculdade de Ciências e Tecnologia, FCT.

Dias, C.G., Batuca, J.R., Marinho, A.T., Caixas, U., Monteiro, E.C., Antunes, A.M.M., Pereira, S.A., 2014b. Quantification of the arylesterase activity of paraoxonase-1 in human blood. Anal. Methods 6, 289.

Díaz-Delfín, J., del Mar Gutiérrez, M., Gallego-Escuredo, J.M., Domingo, J.C., Gracia Mateo, M., Villarroya, F., Domingo, P., Giralt, M., 2011. Effects of nevirapine and efavirenz on human adipocyte differentiation, gene expression, and release of adipokines and cytokines. Antiviral Res. 91, 112–119. https://doi.org/10.1016/j.antiviral.2011.04.018

Dickinson, L., Chaponda, M., Carr, D.F., van Oosterhout, J.J., Kumwenda, J., Lalloo, D.G., Pirmohamed, M., Heyderman, R.S., Khoo, S.H., 2014. Population Pharmacokinetic and Pharmacogenetic Analysis of Nevirapine in Hypersensitive and Tolerant HIV-Infected Patients from Malawi. Antimicrob. Agents Chemother. 58, 706–712. https://doi.org/10.1128/AAC.02069-13

Dilly, S.J., Morris, G.S., 2017. Pimping up Drugs Recovered, Superannuated and Under Exploited Drugs - An Introduction to the Basics of Drug Reprofiling. Curr. Drug Discov. Technol. 14, 121–126. https://doi.org/10.2174/1570163814666170117120005

Dlamini, Z., Hull, R., 2017. Can the HIV-1 splicing machinery be targeted for drug discovery? HIVAIDS - Res. Palliat. Care Volume 9, 63–75. https://doi.org/10.2147/HIV.S120576

Donato, M.T., Gomezlechon, M.J., Castell, J.V., 1993. A Microassay for Measuring Cytochrome P450IA1 and Cytochrome P450IIB1 Activities in Intact Human and Rat Hepatocytes Cultured on 96-Well Plates. Anal. Biochem. 213, 29–33. https://doi.org/10.1006/abio.1993.1381

Donnelly, C.A., Bartley, L.M., Ghani, A.C., Le Fevre, A.M., Kwong, G.P., Cowling, B.J., van Sighem, A.I., de Wolf, F., Rode, R.A., Anderson, R.M., 2005. Gender difference in HIV-1 RNA viral loads. HIV Med. 6, 170–178. https://doi.org/10.1111/j.1468-1293.2005.00285.x

Dooley, T.P., Haldeman-Cahill, R., Joiner, J., Wilborn, T.W., 2000. Expression Profiling of Human Sulfotransferase and Sulfatase Gene Superfamilies in Epithelial Tissues and Cultured Cells. Biochem. Biophys. Res. Commun. 277, 236–245. https://doi.org/10.1006/bbrc.2000.3643

Draganov, D.I., La Du, B.N., 2004. Pharmacogenetics of paraoxonases: a brief review. Naunyn. Schmiedebergs Arch. Pharmacol. 369, 78–88. https://doi.org/10.1007/s00210-003-0833-1

Draganov, D.I., Teiber, J.F., 2008. PONs' Natural Substrates – The Key for their Physiological Roles, in: Mackness, B., Mackness, M., Aviram, M., Paragh, G. (Eds.), The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism. Springer Netherlands, Dordrecht, pp. 297–305. https://doi.org/10.1007/978-1-4020-6561-3_20

Draganov, D.I., Teiber, J.F., Speelman, A., Osawa, Y., Sunahara, R., La Du, B.N., 2005. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. J. Lipid Res. 46, 1239–1247. https://doi.org/10.1194/jlr.M400511-JLR200

Dragovic, G., Smith, C.J., Jevtovic, D., Dimitrijevic, B., Kusic, J., Youle, M., Johnson, M.A., 2016. Choice of first-line antiretroviral therapy regimen and treatment outcomes for HIV in a middle income compared to a high income country: a cohort study. BMC Infect. Dis. 16. https://doi.org/10.1186/s12879-016-1443-0

Duanmu, Z., Locke, D., Smigelski, J., Wu, W., Dahn, M.S., Falany, C.N., Kocarek, T.A., Runge-Morris, M., 2002. Effects of dexamethasone on aryl (SULT1A1)- and hydroxysteroid (SULT2A1)-sulfotransferase gene expression in primary cultured human hepatocytes. Drug Metab. Dispos. Biol. Fate Chem. 30, 997–1004.

Dullaart, R.P.F., Otvos, J.D., James, R.W., 2014. Serum paraoxonase-1 activity is more closely related to HDL particle concentration and large HDL particles than to HDL cholesterol in Type 2 diabetic and non-diabetic subjects. Clin. Biochem. 47, 1022–1027. https://doi.org/10.1016/j.clinbiochem.2014.04.013

Eaton, D.L., Bammler, T.K., 1999. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. Off. J. Soc. Toxicol. 49, 156–164.

Edelson, S., 2010. Going APOA1 in cancer. Sci.-Bus. Exch. 3. https://doi.org/10.1038/scibx.2010.1312

Ehmann, M., Felix, K., Hartmann, D., Schn??lzer, M., Nees, M., Vorderw??lbecke, S., Bogumil, R., B??chler, M.W., Friess, H., 2007. Identification of Potential Markers for the Detection of Pancreatic Cancer Through Comparative Serum Protein Expression Profiling: Pancreas 34, 205–214. https://doi.org/10.1097/01.mpa.0000250128.57026.b2

El-Sadr, W.M., Mullin, C.M., Carr, A., Gibert, C., Rappoport, C., Visnegarwala, F., Grunfeld, C., Raghavan, S.S., 2005. Effects of HIV disease on lipid, glucose and insulin levels: results from a large antiretroviral-naive cohort. HIV Med. 6, 114–121. https://doi.org/10.1111/j.1468-1293.2005.00273.x

Enk, L., Crona, N., Olsson, J.H., Hillensjö, T., 1986. Lipids, apolipoproteins and steroids in serum and in fluid from stimulated and non-stimulated human ovarian follicles. Acta Endocrinol. (Copenh.) 111, 558–562.

Erickson, D.A., Mather, G., Trager, W.F., Levy, R.H., Keirns, J.J., 1999. Characterization of the in vitro biotransformation of the HIV-1 reverse transcriptase inhibitor nevirapine by human hepatic cytochromes P-450. Drug Metab. Dispos. Biol. Fate Chem. 27, 1488–1495.

Eyvazian, V.A., Frishman, W.H., 2017. Evacetrapib: Another CETP Inhibitor for Dyslipidemia with No Clinical Benefit. Cardiol. Rev. 1. https://doi.org/10.1097/CRD.0000000000000137

Fadini, G.P., Bonora, B.M., Zatti, G., Vitturi, N., Iori, E., Marescotti, M.C., Albiero, M., Avogaro, A., 2017. Effects of the SGLT2 inhibitor dapagliflozin on HDL cholesterol, particle size, and cholesterol efflux capacity in patients with type 2 diabetes: a randomized placebo-controlled trial. Cardiovasc. Diabetol. 16. https://doi.org/10.1186/s12933-017-0529-3

Falany, C.N., Rohn-Glowacki, K.J., 2013. SULT2B1: unique properties and characteristics of a hydroxysteroid sulfotransferase family. Drug Metab. Rev. 45, 388–400. https://doi.org/10.3109/03602532.2013.835609

Fan, Q., Cai, Q., Xu, Y., 2015. FOXM1 is a downstream target of LPA and YAP oncogenic signaling pathways in high grade serous ovarian cancer. Oncotarget 6, 27688–27699. https://doi.org/10.18632/oncotarget.4280

Fang, H.-L., Strom, S.C., Cai, H., Falany, C., Kocarek, T.A., Runge-Morris, M., 2005. Regulation of Human Hepatic Hydroxysteroid Sulfotransferase Gene Expression by the Peroxisome Proliferator-Activated Receptor Transcription Factor. Mol. Pharmacol. 67, 1257–1267. https://doi.org/10.1124/mol.104.005389

Fang, J.-L., Beland, F.A., 2013. Differential responses of human hepatocytes to the non-nucleoside HIV-1 reverse transcriptase inhibitor nevirapine. J. Toxicol. Sci. 38, 741–752.

Fang, X., Schummer, M., Mao, M., Yu, S., Tabassam, F.H., Swaby, R., Hasegawa, Y., Tanyi, J.L., LaPushin, R., Eder, A., Jaffe, R., Erickson, J., Mills, G.B., 2002. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. Biochim. Biophys. Acta 1582, 257–264.

Fan-Havard, P., Liu, Z., Chou, M., Ling, Y., Barrail-Tran, A., Haas, D.W., Taburet, A.-M., the ANRS12154 Study Group, 2013. Pharmacokinetics of Phase I Nevirapine Metabolites

following a Single Dose and at Steady State. Antimicrob. Agents Chemother. 57, 2154–2160. https://doi.org/10.1128/AAC.02294-12

Faucette, S.R., Zhang, T.-C., Moore, R., Sueyoshi, T., Omiecinski, C.J., LeCluyse, E.L., Negishi, M., Wang, H., 2006. Relative Activation of Human Pregnane X Receptor versus Constitutive Androstane Receptor Defines Distinct Classes of CYP2B6 and CYP3A4 Inducers. J. Pharmacol. Exp. Ther. 320, 72–80. https://doi.org/10.1124/jpet.106.112136

Ferreira, A.M., Marques da Silva, P., 2016. Defining the Place of Ezetimibe/Atorvastatin in the Management of Hyperlipidemia. Am. J. Cardiovasc. Drugs. https://doi.org/10.1007/s40256-016-0205-0

Ferri, N., Corsini, A., Sirtori, C., Ruscica, M., 2017. PPAR-α agonists are still on the rise: an update on clinical and experimental findings. Expert Opin. Investig. Drugs 1–10. https://doi.org/10.1080/13543784.2017.1312339

Floridia, M., Tamburrini, E., Anzidei, G., Tibaldi, C., Guaraldi, G., Guerra, B., Meloni, A., Vimercati, A., Molinari, A., Pinnetti, C., Dalzero, S., Ravizza, M., 2009. Plasma Lipid Profile in Pregnant Women with HIV Receiving Nevirapine. AIDS Patient Care STDs 23, 147–152. https://doi.org/10.1089/apc.2008.0148

Foley, O.W., Rauh-Hain, J.A., del Carmen, M.G., 2013. Recurrent epithelial ovarian cancer: an update on treatment. Oncol. Williston Park N 27, 288–294, 298.

Franssen, R., Sankatsing, R.R., Hassink, E., Hutten, B., Ackermans, M.T., Brinkman, K., Oesterholt, R., Arenas-Pinto, A., Storfer, S.P., Kastelein, J.J., Sauerwein, H.P., Reiss, P., Stroes, E.S., 2009. Nevirapine Increases High-Density Lipoprotein Cholesterol Concentration by Stimulation of Apolipoprotein A-I Production. Arterioscler. Thromb. Vasc. Biol. 29, 1336–1341. https://doi.org/10.1161/ATVBAHA.109.192088

Fraser, M., Bai, T., Tsang, B.K., 2008. Akt promotes cisplatin resistance in human ovarian cancer cells through inhibition of p53 phosphorylation and nuclear function. Int. J. Cancer 122, 534–546. https://doi.org/10.1002/ijc.23086

Fuda, H., Shimizu, C., Lee, Y.C., Akita, H., Strott, C.A., 2002. Characterization and expression of human bifunctional 3'-phosphoadenosine 5'-phosphosulphate synthase isoforms. Biochem. J. 365, 497–504. https://doi.org/10.1042/bj20020044

Fumero, E., Podzamczer, D., 2001. The role of nevirapine in the treatment of HIV-1 disease. Expert Opin. Pharmacother. 2, 2065–2078. https://doi.org/10.1517/14656566.2.12.2065

Gabra, H., Stronach, E., Rohini, S., 2008. Molecular biology., in: Treatment of Cancer. CRC Press, pp. 23–36. https://doi.org/10.1201/b13550

Gadkar, K., Lu, J., Sahasranaman, S., Davis, J., Mazer, N.A., Ramanujan, S., 2016. Evaluation of HDL-modulating interventions for cardiovascular risk reduction using a systems pharmacology approach. J. Lipid Res. 57, 46–55. https://doi.org/10.1194/jlr.M057943

Gaidukov, L., R. I, V., Yacobson, S., Rosenblat, M., Aviram, M., Tawfik, D.S., 2010. ApoE Induces Serum Paraoxonase PON1 Activity and Stability Similar to ApoA-I. Biochemistry (Mosc.) 49, 532–538. https://doi.org/10.1021/bi9013227

Gallagher, C.J., Balliet, R.M., Sun, D., Chen, G., Lazarus, P., 2010. Sex Differences in UDP-Glucuronosyltransferase 2B17 Expression and Activity. Drug Metab. Dispos. 38, 2204–2209. https://doi.org/10.1124/dmd.110.035345

Gamage, N., 2005. Human Sulfotransferases and Their Role in Chemical Metabolism. Toxicol. Sci. 90, 5–22. https://doi.org/10.1093/toxsci/kfj061

Ganapathy, E., Su, F., Meriwether, D., Devarajan, A., Grijalva, V., Gao, F., Chattopadhyay, A., Anantharamaiah, G.M., Navab, M., Fogelman, A.M., Reddy, S.T., Farias-Eisner, R., 2012. D-4F, an apoA-I mimetic peptide, inhibits proliferation and tumorigenicity of epithelial ovarian cancer cells by upregulating the antioxidant enzyme MnSOD. Int. J. Cancer 130, 1071–1081. https://doi.org/10.1002/ijc.26079

Gandhi, M., Bacchetti, P., Miotti, P., Quinn, T.C., Veronese, F., Greenblatt, R.M., 2002. Does Patient Sex Affect Human Immunodeficiency Virus Levels? Clin. Infect. Dis. 35, 313–322. https://doi.org/10.1086/341249

Gao, F., Chattopadhyay, A., Navab, M., Grijalva, V., Su, F., Fogelman, A.M., Reddy, S.T., Farias-Eisner, R., 2012. Apolipoprotein A-I Mimetic Peptides Inhibit Expression and Activity of Hypoxia-Inducible Factor-1 in Human Ovarian Cancer Cell Lines and a Mouse Ovarian Cancer Model. J. Pharmacol. Exp. Ther. 342, 255–262. https://doi.org/10.1124/jpet.112.191544

Gao, F., Vasquez, S.X., Su, F., Roberts, S., Shah, N., Grijalva, V., Imaizumi, S., Chattopadhyay, A., Ganapathy, E., Meriwether, D., Johnston, B., Anantharamaiah, G.M., Navab, M., Fogelman, A.M., Reddy, S.T., Farias-Eisner, R., 2011. L-5F, an apolipoprotein A-I mimetic, inhibits tumor angiogenesis by suppressing VEGF/basic FGF signaling pathways. Integr. Biol. 3, 479. https://doi.org/10.1039/c0ib00147c

Gaus, K., Kritharides, L., Schmitz, G., Boettcher, A., Drobnik, W., Langmann, T., Quinn, C., Death, A., Dean, R., Jessup, W., 2004. Apolipoprotein A-1 interaction with plasma membrane lipid rafts controls cholesterol export from macrophages. FASEB J. https://doi.org/10.1096/fj.03-0486fje

Gavi, S., Shumay, E., Wang, H., Malbon, C.C., 2006. G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. Trends Endocrinol. Metab. 17, 48–54. https://doi.org/10.1016/j.tem.2006.01.006

Geese, W.J., Blanchard Raftogianis, R., 2001. Biochemical Characterization and Tissue Distribution of Human SULT2B1. Biochem. Biophys. Res. Commun. 288, 280–289. https://doi.org/10.1006/bbrc.2001.5746

Gerets, H.H.J., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B.O., Dhalluin, S., Atienzar, F.A., 2012. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol. Toxicol. 28, 69–87. https://doi.org/10.1007/s10565-011-9208-4

German, J.B., Smilowitz, J.T., Zivkovic, A.M., 2006. Lipoproteins: When size really matters. Curr. Opin. Colloid Interface Sci. 11, 171–183. https://doi.org/10.1016/j.cocis.2005.11.006

Geyer, J., Bakhaus, K., Bernhardt, R., Blaschka, C., Dezhkam, Y., Fietz, D., Grosser, G., Hartmann, K., Hartmann, M.F., Neunzig, J., Papadopoulos, D., Sánchez-Guijo, A., Scheiner-Bobis, G., Schuler, G., Shihan, M., Wrenzycki, C., Wudy, S.A., Bergmann, M., 2016. The role of sulfated steroid hormones in reproductive processes. J. Steroid Biochem. Mol. Biol. https://doi.org/10.1016/j.jsbmb.2016.07.002

Gkouskou, K.K., Ioannou, M., Pavlopoulos, G.A., Georgila, K., Siganou, A., Nikolaidis, G., Kanellis, D.C., Moore, S., Papadakis, K.A., Kardassis, D., Iliopoulos, I., McDyer, F.A., Drakos, E., Eliopoulos, A.G., 2016. Apolipoprotein A-I inhibits experimental colitis and colitis-propelled carcinogenesis. Oncogene 35, 2496–2505. https://doi.org/10.1038/onc.2015.307

Gleason, R.L., Caulk, A.W., Seifu, D., Rosebush, J.C., Shapiro, A.M., Schwartz, M.H., Eckard, A.R., Amogne, W., Abebe, W., 2016. Efavirenz and ritonavir-boosted lopinavir use exhibited elevated markers of atherosclerosis across age groups in people living with HIV in Ethiopia. J. Biomech. 49, 2584–2592. https://doi.org/10.1016/j.jbiomech.2016.05.018

Gomes, A.R., Zhao, F., Lam, E.W.F., 2013. Role and regulation of the forkhead transcription factors FOXO3a and FOXM1 in carcinogenesis and drug resistance. Chin. J. Cancer 32, 365–370. https://doi.org/10.5732/cjc.012.10277

González, M., 2017. The HIV-1 Vpr Protein: A Multifaceted Target for Therapeutic Intervention. Int. J. Mol. Sci. 18, 126. https://doi.org/10.3390/ijms18010126

Gordon, D.J., Probstfield, J.L., Garrison, R.J., Neaton, J.D., Castelli, W.P., Knoke, J.D., Jacobs, D.R., Bangdiwala, S., Tyroler, H.A., 1989. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 79, 8–15.

Gouedard, C., Barouki, R., Morel, Y., 2004a. Dietary Polyphenols Increase Paraoxonase 1 Gene Expression by an Aryl Hydrocarbon Receptor-Dependent Mechanism. Mol. Cell. Biol. 24, 5209–5222. https://doi.org/10.1128/MCB.24.12.5209-5222.2004

Gouedard, C., Barouki, R., Morel, Y., 2004b. Induction of the Paraoxonase-1 Gene Expression by Resveratrol. Arterioscler. Thromb. Vasc. Biol. 24, 2378–2383. https://doi.org/10.1161/01.ATV.0000146530.24736.ce

Grillo, M.P., 2015. Detecting reactive drug metabolites for reducing the potential for drug toxicity. Expert Opin. Drug Metab. Toxicol. 11, 1281–1302. https://doi.org/10.1517/17425255.2015.1048222

Grilo, N.M., Marinho, A.T., Naranjo, M.E.G., Caixas, U., Branco, T., Antunes, A.M.M., Marques, M.M., Monteiro, E.C., Llerena, A., Pereira, S.A., 2013. Relevance of CYP2C19 genotypes in Neviperapine biotransformation. Second ESPT Conference "Pharmacogenomics: From Cell to Clinic," in: Drug Metabolism and Drug Interactions. https://doi.org/10.1515/dmdi-2013-0041

Gugliucci, A., Caccavello, R., Nassar, H., Ahmad, W.A., Sinnreich, R., Kark, J.D., 2015. Low protective PON1 lactonase activity in an Arab population with high rates of coronary heart disease and diabetes. Clin. Chim. Acta 445, 41–47. https://doi.org/10.1016/j.cca.2015.03.012

Gungor, H., Saleem, A., Babar, S., Dina, R., El-Bahrawy, M.A., Curry, E., Rama, N., Chen, M., Pickford, E., Agarwal, R., Blagden, S., Carme, S., Salinas, C., Madison, S., Krachey, E., Santiago-Walker, A., Smith, D.A., Morris, S.R., Stronach, E.A., Gabra, H., 2015. Dose-Finding Quantitative 18F-FDG PET Imaging Study with the Oral Pan-AKT Inhibitor GSK2141795 in Patients with Gynecologic Malignancies. J. Nucl. Med. 56, 1828–1835. https://doi.org/10.2967/jnumed.115.156505

Günthard, H.F., Aberg, J.A., Eron, J.J., Hoy, J.F., Telenti, A., Benson, C.A., Burger, D.M., Cahn, P., Gallant, J.E., Glesby, M.J., Reiss, P., Saag, M.S., Thomas, D.L., Jacobsen, D.M., Volberding, P.A., 2014. Antiretroviral Treatment of Adult HIV Infection: 2014 Recommendations of the International Antiviral Society–USA Panel. JAMA 312, 410. https://doi.org/10.1001/jama.2014.8722

Guo, S., He, X., Chen, Q., Yang, G., Yao, K., Dong, P., Ye, Y., Chen, D., Zhang, Z., Qin, Z., Liu, Z., Li, Z., Xue, Y., Zhang, M., Liu, R., Zhou, F., Han, H., 2016. The Effect of Preoperative Apolipoprotein A-I on the Prognosis of Surgical Renal Cell Carcinoma: A Retrospective Large Sample Study. Medicine (Baltimore) 95, e3147. https://doi.org/10.1097/MD.0000000000003147

Guyot, E., Coumoul, X., Chassé, J.-F., Khallouki, F., Savouret, J.F., Poirot, M., Barouki, R., 2012. Identification of a new stilbene-derived inducer of paraoxonase 1 and ligand of the Aryl hydrocarbon Receptor. Biochem. Pharmacol. 83, 627–632. https://doi.org/10.1016/j.bcp.2011.12.013

Hafez, M.M., Al-Shabanah, O.A., Al-Harbi, N.O., Al-Harbi, M.M., Al-Rejaie, S.S., Alsurayea, S.M., Sayed-Ahmed, M.M., 2014. Association between Paraoxonases Gene Expression and Oxidative Stress in Hepatotoxicity Induced by CCl_4. Oxid. Med. Cell. Longev. 2014, 1–12. https://doi.org/10.1155/2014/893212

Hahne, J.C., Honig, A., Engel, J.B., Lampis, A., Valeri, N., 2016. Analysing Molecular Mechanism Related to Therapy- Resistance in In-vitro Models of Ovarian Cancer, in: Farghaly, S.A. (Ed.), Gynecologic Cancers - Basic Sciences, Clinical and Therapeutic Perspectives. InTech. https://doi.org/10.5772/60727

Hammad, M.A., Abdel-Bakky, M.S., Walker, L.A., Ashfaq, M.K., 2011. Oxidized low-density lipoprotein and tissue factor are involved in monocrotaline/lipopolysaccharide-induced hepatotoxicity. Arch. Toxicol. 85, 1079–1089. https://doi.org/10.1007/s00204-011-0649-6

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of Cancer: The Next Generation. Cell 144, 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Harel, M., Aharoni, A., Gaidukov, L., Brumshtein, B., Khersonsky, O., Meged, R., Dvir, H., Ravelli, R.B.G., McCarthy, A., Toker, L., Silman, I., Sussman, J.L., Tawfik, D.S., 2004. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat. Struct. Mol. Biol. 11, 412–419. https://doi.org/10.1038/nsmb767

Harjivan, S.G., Pinheiro, P.F., Martins, I.L., Godinho, A.L., Wanke, R., Santos, P.P., Pereira, S.A., Beland, F.A., Marques, M.M., Antunes, A.M.M., 2015. Quinoid derivatives of the nevirapine metabolites 2-hydroxy- and 3-hydroxy-nevirapine: activation pathway to amino acid adducts. Toxicol Res 4, 1565–1577. https://doi.org/10.1039/C5TX00176E

Hassan, H.H., Denis, M., Krimbou, L., Marcil, M., Genest, J., 2006. Cellular cholesterol homeostasis in vascular endothelial cells. Can. J. Cardiol. 22 Suppl B, 35B–40B.

Havlir, D., Cheeseman, S.H., McLaughlin, M., Murphy, R., Erice, A., Spector, S.A., Greenough, T.C., Sullivan, J.L., Hall, D., Myers, M., 1995. High-dose nevirapine: safety, pharmacokinetics, and antiviral effect in patients with human immunodeficiency virus infection. J. Infect. Dis. 171, 537–545.

Hecht, M., Erber, S., Harrer, T., Klinker, H., Roth, T., Parsch, H., Fiebig, N., Fietkau, R., Distel, L.V., 2015. Efavirenz Has the Highest Anti-Proliferative Effect of Non-Nucleoside Reverse Transcriptase Inhibitors against Pancreatic Cancer Cells. PLOS ONE 10, e0130277. https://doi.org/10.1371/journal.pone.0130277

Heinecke, J.W., 2012. The not-so-simple HDL story: A new era for quantifying HDL and cardiovascular risk? Nat. Med. 18, 1346–1347. https://doi.org/10.1038/nm.2930

Hempel, N., Gamage, N., Martin, J.L., McManus, M.E., 2007. Human cytosolic sulfotransferase SULT1A1. Int. J. Biochem. Cell Biol. 39, 685–689. https://doi.org/10.1016/j.biocel.2006.10.002

Hermann, T., 2016. Small molecules targeting viral RNA: Small molecules targeting viral RNA. Wiley Interdiscip. Rev. RNA 7, 726–743. https://doi.org/10.1002/wrna.1373

Hirao, J., Nishimura, M., Arakawa, S., Niino, N., Mori, K., Furukawa, T., Sanbuissho, A., Manabe, S., Nishihara, M., Mori, Y., 2011. Sex and circadian modulatory effects on rat liver as assessed by transcriptome analyses. J. Toxicol. Sci. 36, 9–22.

Ho, T.T., Wong, K.H., Chan, K.C., Lee, S.S., 1998. High incidence of nevirapine-associated rash in HIV-infected Chinese. AIDS Lond. Engl. 12, 2082–2083.

House, R.V., Luster, M.I., Dean, J.H., Johnson, V.J., 2014. Immunotoxicology: The Immune System Response to Toxic Insult., in: Hayes, A.W., Kruger, C.L. (Eds.), Hayes' Principles and Methods of Toxicology. CRC Press.

Hsue, P.Y., Hunt, P.W., Schnell, A., Kalapus, S.C., Hoh, R., Ganz, P., Martin, J.N., Deeks, S.G., 2009. Role of viral replication, antiretroviral therapy, and immunodeficiency in HIV-associated atherosclerosis: AIDS 23, 1059–1067. https://doi.org/10.1097/QAD.0b013e32832b514b

Hu, X., Li, D., Zhang, W., Zhou, J., Tang, B., Li, L., 2012. Matrix metalloproteinase-9 expression correlates with prognosis and involved in ovarian cancer cell invasion. Arch. Gynecol. Obstet. 286, 1537–1543. https://doi.org/10.1007/s00404-012-2456-6

Hu, Y.L., Tee, M.K., Goetzl, E.J., Auersperg, N., Mills, G.B., Ferrara, N., Jaffe, R.B., 2001. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. J. Natl. Cancer Inst. 93, 762–768.

Huang, H., Tindall, D.J., 2011. Regulation of FOXO protein stability via ubiquitination and proteasome degradation. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1813, 1961–1964. https://doi.org/10.1016/j.bbamcr.2011.01.007

Huang, H.-L., Stasyk, T., Morandell, S., Dieplinger, H., Falkensammer, G., Griesmacher, A., Mogg, M., Schreiber, M., Feuerstein, I., Huck, C.W., Stecher, G., Bonn, G.K., Huber, L.A., 2006. Biomarker discovery in breast cancer serum using 2-D differential gel electrophoresis/ MALDI-TOF/TOF and data validation by routine clinical assays. ELECTROPHORESIS 27, 1641–1650. https://doi.org/10.1002/elps.200500857

Huang, J.T.-J., Wang, L., Prabakaran, S., Wengenroth, M., Lockstone, H.E., Koethe, D., Gerth, C.W., Gross, S., Schreiber, D., Lilley, K., Wayland, M., Oxley, D., Leweke, F.M., Bahn, S., 2008. Independent protein-profiling studies show a decrease in apolipoprotein A1 levels in schizophrenia CSF, brain and peripheral tissues. Mol. Psychiatry 13, 1118–1128. https://doi.org/10.1038/sj.mp.4002108

Huang, Y., DiDonato, J.A., Levison, B.S., Schmitt, D., Li, L., Wu, Y., Buffa, J., Kim, T., Gerstenecker, G.S., Gu, X., Kadiyala, C.S., Wang, Z., Culley, M.K., Hazen, J.E., DiDonato, A.J., Fu, X., Berisha, S.Z., Peng, D., Nguyen, T.T., Liang, S., Chuang, C.-C., Cho, L., Plow, E.F., Fox, P.L., Gogonea, V., Tang, W.H.W., Parks, J.S., Fisher, E.A., Smith, J.D., Hazen, S.L., 2014. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nat. Med. 20, 193–203. https://doi.org/10.1038/nm.3459

Huntington, S., Thorne, C., Anderson, J., Newell, M.-L., Taylor, G., Pillay, D., Hill, T., Tookey, P., Sabin, C., 2014. Does pregnancy increase the risk of ART-induced hepatotoxicity among HIV-positive women? J. Int. AIDS Soc. 17. https://doi.org/10.7448/IAS.17.4.19486

Ibarra, M., Vázquez, M., Fagiolino, P., 2014. Population pharmacokinetic model to analyze nevirapine multiple-peaks profile after a single oral dose. J. Pharmacokinet. Pharmacodyn. 41, 363–373. https://doi.org/10.1007/s10928-014-9371-3

Iqbal, A.J., Barrett, T.J., Taylor, L., McNeill, E., Manmadhan, A., Recio, C., Carmineri, A., Brodermann, M.H., White, G.E., Cooper, D., DiDonato, J.A., Zamanian-Daryoush, M., Hazen, S.L., Channon, K.M., Greaves, D.R., Fisher, E.A., 2016. Acute exposure to apolipoprotein A1 inhibits macrophage chemotaxis in vitro and monocyte recruitment in vivo. eLife 5. https://doi.org/10.7554/eLife.15190

Iqbal, F., Baker, W.S., Khan, M.I., Thukuntla, S., McKinney, K.H., Abate, N., Tuvdendorj, D., 2017. Current and future therapies for addressing the effects of inflammation on HDL cholesterol metabolism: Inflammation and HDL-C metabolism. Br. J. Pharmacol. https://doi.org/10.1111/bph.13743

Irusta, G., Abramovich, D., Parborell, F., Tesone, M., 2010. Direct survival role of vascular endothelial growth factor (VEGF) on rat ovarian follicular cells. Mol. Cell. Endocrinol. 325, 93–100. https://doi.org/10.1016/j.mce.2010.04.018

Ishibashi, S., Yamashita, S., Arai, H., Araki, E., Yokote, K., Suganami, H., Fruchart, J.-C., Kodama, T., 2016. Effects of K-877, a novel selective PPARα modulator (SPPARMα), in dyslipidaemic patients: A randomized, double blind, active- and placebo-controlled, phase 2 trial. Atherosclerosis 249, 36–43. https://doi.org/10.1016/j.atherosclerosis.2016.02.029

Ishida, B.Y., Albee, D., Paigen, B., 1990. Interconversion of prebeta-migrating lipoproteins containing apolipoprotein A-I and HDL. J. Lipid Res. 31, 227–236.

Jaichander, P., Selvarajan, K., Garelnabi, M., Parthasarathy, S., 2008. Induction of paraoxonase 1 and apolipoprotein A-I gene expression by aspirin. J. Lipid Res. 49, 2142–2148. https://doi.org/10.1194/jlr.M800082-JLR200

Jakubowski, H., 2006. Pathophysiological consequences of homocysteine excess. J. Nutr. 136, 1741S–1749S.

Jakubowski, H., 2000. Calcium-dependent human serum homocysteine thiolactone hydrolase. A protective mechanism against protein N-homocysteinylation. J. Biol. Chem. 275, 3957–3962.

Jakubowski, H., 1999. Protein homocysteinylation: possible mechanism underlying pathological consequences of elevated homocysteine levels. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 13, 2277–2283.

Jarvik, G.P., Rozek, L.S., Brophy, V.H., Hatsukami, T.S., Richter, R.J., Schellenberg, G.D., Furlong, C.E., 2000. Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1(192) or PON1(55) genotype. Arterioscler. Thromb. Vasc. Biol. 20, 2441–2447.

Jeon, E.S., Heo, S.C., Lee, I.H., Choi, Y.J., Park, J.H., Choi, K.U., Park, D.Y., Suh, D.-S., Yoon, M.-S., Kim, J.H., 2010. Ovarian cancer-derived lysophosphatidic acid stimulates secretion of VEGF and stromal cell-derived factor-1α from human mesenchymal stem cells. Exp. Mol. Med. 42, 280. https://doi.org/10.3858/emm.2010.42.4.027

Jeong, G.O., Shin, S.H., Seo, E.J., Kwon, Y.W., Heo, S.C., Kim, K.-H., Yoon, M.-S., Suh, D.-S., Kim, J.H., 2013. TAZ Mediates Lysophosphatidic Acid-Induced Migration and Proliferation of Epithelial Ovarian Cancer Cells. Cell. Physiol. Biochem. 32, 253–263. https://doi.org/10.1159/000354434

Jeong, T.C., Chang, H.W., Lee, E.S., Jeon, T.W., Jeong, H.G., Holsapple, M.P., 2004. SKF 525-A INDUCES COCAINE *N* -DEMETHYLASE, ETHOXYRESORUFIN *O* -DEETHYLASE, AND PENTOXYRESORUFIN *O* -DEALKYLASE ACTIVITIES BY INDUCTION OF CYTOCHROME P-450 2B IN FEMALE B6C3F1 MICE. J. Toxicol. Environ. Health A 67, 1955–1970. https://doi.org/10.1080/15287390490514606

Jia, Z.-H., Jia, Y., Guo, F.-J., Chen, J., Zhang, X.-W., Cui, M.-H., 2017. Phosphorylation of STAT3 at Tyr705 regulates MMP-9 production in epithelial ovarian cancer. PLOS ONE 12, e0183622. https://doi.org/10.1371/journal.pone.0183622

Jian, Z.-H., Lung, C.-C., Ko, P.-C., Sun, Y.-H., Huang, J.-Y., Ho, C.-C., Ho, C.-Y., Chiang, Y.-C., Chen, C.-J., Liaw, Y.-P., 2013. The association between the apolipoprotein A1/ high density lipoprotein -cholesterol and diabetes in Taiwan — a cross-sectional study. BMC Endocr. Disord. 13. https://doi.org/10.1186/1472-6823-13-42

Johnson, M.S.C., Svensson, P.-A., Helou, K., Billig, H., Levan, G., Carlsson, L.M.S., Carlsson, B., 1998. Characterization and Chromosomal Localization of Rat Scavenger Receptor Class B Type I, a High Density Lipoprotein Receptor with a Putative Leucine Zipper Domain and Peroxisomal Targeting Sequence [1]. Endocrinology 139, 72–80. https://doi.org/10.1210/endo.139.1.5666

Joshi, P.H., Toth, P.P., Lirette, S.T., Griswold, M.E., Massaro, J.M., Martin, S.S., Blaha, M.J., Kulkarni, K.R., Khokhar, A.A., Correa, A., D'Agustino, R.B., Jones, S.R., 2016. Association of high-density lipoprotein subclasses and incident coronary heart disease: The Jackson Heart and Framingham Offspring Cohort Studies. Eur. J. Prev. Cardiol. 23, 41–49. https://doi.org/10.1177/2047487314543890

Jung, Y.S., Ryu, S., Chang, Y., Yun, K.E., Park, J.H., Kim, H.J., Cho, Y.K., Sohn, C.I., Jeon, W.K., Kim, B.I., Choi, K., Park, D.I., 2015. Associations Between Parameters of Glucose and Lipid Metabolism and Risk of Colorectal Neoplasm. Dig. Dis. Sci. 60, 2996–3004. https://doi.org/10.1007/s10620-015-3713-x

Kajinami, K., Brousseau, M.E., Lamon-Fava, S., Ordovas, J.M., Schaefer, E.J., 2005. Gender-specific effects of estrogen receptor alpha gene haplotype on high-density lipoprotein cholesterol response to atorvastatin: interaction with apolipoprotein AI gene polymorphism. Atherosclerosis 178, 331–338. https://doi.org/10.1016/j.atherosclerosis.2004.08.034

Kamila, B., M, S.D., Hieronim, J., 2012. Metabolism and Neurotoxicity of Homocysteine Thiolactone in Mice: Evidence for a Protective Role of Paraoxonase 1. J. Alzheimeraposs Dis. 225–231. https://doi.org/10.3233/JAD-2012-111940

Kang, D., Huo, Z., Wu, G., Xu, J., Zhan, P., Liu, X., 2017. Novel fused pyrimidine and isoquinoline derivatives as potent HIV-1 NNRTIs: a patent evaluation of WO2016105532A1, WO2016105534A1 and WO2016105564A1. Expert Opin. Ther. Pat. 27, 383–391. https://doi.org/10.1080/13543776.2017.1303046

Kannisto, K., Gåfvels, M., Jiang, Z.-Y., Slätis, K., Hu, X., Jorns, C., Steffensen, K.R., Eggertsen, G., 2014. LXR Driven Induction of HDL-Cholesterol is Independent of Intestinal Cholesterol Absorption and ABCA1 Protein Expression. Lipids 49, 71–83. https://doi.org/10.1007/s11745-013-3853-8

Karaman, E., Uzun, H., Papila, I., Balci, H., Ozdilek, A., Genc, H., Yanardag, H., Papila, C., 2010. Serum Paraoxonase Activity and Oxidative DNA Damage in Patients With Laryngeal

Squamous Cell Carcinoma: J. Craniofac. Surg. 21, 1745–1749. https://doi.org/10.1097/SCS.0b013e3181f4040a

Karavia, E.A., Papachristou, D.J., Liopeta, K., Triantaphyllidou, I.-E., Dimitrakopoulos, O., Kypreos, K.E., 2012. Apolipoprotein A-I modulates processes associated with diet-induced nonalcoholic fatty liver disease in mice. Mol. Med. Camb. Mass 18, 901–912. https://doi.org/10.2119/molmed.2012.00113

Karlsson, H., Kontush, A., James, R.W., 2015. Functionality of HDL: Antioxidation and Detoxifying Effects, in: von Eckardstein, A., Kardassis, D. (Eds.), High Density Lipoproteins. Springer International Publishing, Cham, pp. 207–228. https://doi.org/10.1007/978-3-319-09665-0_5

Kasko, M., Gaspar, L., Dukát, A., Gavorník, P., Oravec, S., 2014. High-density lipoprotein profile in newly-diagnosed lower extremity artery disease in Slovak population without diabetes mellitus. Neuro Endocrinol. Lett. 35, 531–535.

Kawamoto, T., Kakizaki, S., Yoshinari, K., Negishi, M., 2000. Estrogen Activation of the Nuclear Orphan Receptor CAR (Constitutive Active Receptor) in Induction of the Mouse *Cyp2b10* Gene. Mol. Endocrinol. 14, 1897–1905. https://doi.org/10.1210/mend.14.11.0547

Keane, N.M., Pavlos, R.K., McKinnon, E., Lucas, A., Rive, C., Blyth, C.C., Dunn, D., Lucas, M., Mallal, S., Phillips, E., 2014. HLA Class I restricted CD8+ and Class II restricted CD4+ T cells are implicated in the pathogenesis of nevirapine hypersensitivity: AIDS 28, 1891–1901. https://doi.org/10.1097/QAD.0000000000000345

Kennedy, M.A., Barrera, G.C., Nakamura, K., Baldán, Á., Tarr, P., Fishbein, M.C., Frank, J., Francone, O.L., Edwards, P.A., 2005. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab. 1, 121–131. https://doi.org/10.1016/j.cmet.2005.01.002

Kesselring, A.M., Wit, F.W., Sabin, C.A., Lundgren, J.D., Gill, M.J., Gatell, J.M., Rauch, A., Montaner, J.S., de Wolf, F., Reiss, P., Mocroft, A., 2009. Risk factors for treatment-limiting toxicities in patients starting nevirapine-containing antiretroviral therapy: AIDS 23, 1689–1699. https://doi.org/10.1097/QAD.0b013e32832d3b54

Kessenbrock, K., Plaks, V., Werb, Z., 2010. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. Cell 141, 52–67. https://doi.org/10.1016/j.cell.2010.03.015

Khateeb, J., Gantman, A., Kreitenberg, A.J., Aviram, M., Fuhrman, B., 2010. Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by pomegranate polyphenols: A role for PPAR-γ pathway. Atherosclerosis 208, 119–125. https://doi.org/10.1016/j.atherosclerosis.2009.08.051

Khersonsky, O., Tawfik, D.S., 2005. Structure−Reactivity Studies of Serum Paraoxonase PON1 Suggest that Its Native Activity Is Lactonase †. Biochemistry (Mosc.) 44, 6371–6382. https://doi.org/10.1021/bi047440d

Khodadadi, S., Riazi, G.H., Ahmadian, S., Hoveizi, E., Karima, O., Aryapour, H., 2012. Effect of *N* -homocysteinylation on physicochemical and cytotoxic properties of amyloid β-peptide. FEBS Lett. 586, 127–131. https://doi.org/10.1016/j.febslet.2011.12.018

Kiertiburanakul, S., Sungkanuparph, S., Charoenyingwattana, A., Mahasirimongkol, S., Sura, T., Chantratita, W., 2008. Risk factors for nevirapine-associated rash among HIV-infected patients with low CD4 cell counts in resource-limited settings. Curr. HIV Res. 6, 65–69.

Kim, C., Lee, J., Park, S.-W., Kim, K., Lee, M. won, Paik, S., Jang, A. soo, Kim, D.J., Uh, S., Kim, Y., Park, C., 2016. Attenuation of Cigarette Smoke–Induced Emphysema in Mice by Apolipoprotein A-1 Overexpression. Am. J. Respir. Cell Mol. Biol. 54, 91–102. https://doi.org/10.1165/rcmb.2014-0305OC

Kim, D.S., Marsillach, J., Furlong, C.E., Jarvik, G.P., 2013. Pharmacogenetics of paraoxonase activity: elucidating the role of high-density lipoprotein in disease. Pharmacogenomics 14, 1495–1515. https://doi.org/10.2217/pgs.13.147

Kim, K., Bloom, M.S., Fujimoto, V.Y., Browne, R.W., 2017. Associations between PON1 enzyme activities in human ovarian follicular fluid and serum specimens. PLOS ONE 12, e0172193. https://doi.org/10.1371/journal.pone.0172193

Kingwell, B.A., Chapman, M.J., Kontush, A., Miller, N.E., 2014. HDL-targeted therapies: progress, failures and future. Nat. Rev. Drug Discov. 13, 445–464. https://doi.org/10.1038/nrd4279

Kirbas, A., Kirbas, S., Cure, M.C., Tufekci, A., 2014. Paraoxonase and arylesterase activity and total oxidative/anti-oxidative status in patients with idiopathic Parkinson's disease. J. Clin. Neurosci. 21, 451–455. https://doi.org/10.1016/j.jocn.2013.04.025

Kodydkova, J., Vavrova, L., Stankova, B., Macasek, J., Krechler, T., Zak, A., 2013. Antioxidant Status and Oxidative Stress Markers in Pancreatic Cancer and Chronic Pancreatitis: Pancreas 42, 614–621. https://doi.org/10.1097/MPA.0b013e318288360a

Kontush, A., 2006. Functionally Defective High-Density Lipoprotein: A New Therapeutic Target at the Crossroads of Dyslipidemia, Inflammation, and Atherosclerosis. Pharmacol. Rev. 58, 342–374. https://doi.org/10.1124/pr.58.3.1

Kontush, A., Lindahl, M., Lhomme, M., Calabresi, L., Chapman, M.J., Davidson, W.S., 2015. Structure of HDL: Particle Subclasses and Molecular Components, in: von Eckardstein, A., Kardassis, D. (Eds.), High Density Lipoproteins. Springer International Publishing, Cham, pp. 3–51. https://doi.org/10.1007/978-3-319-09665-0_1

Korkmaz, H., Tabur, S., Özkaya, M., Aksoy, N., Yildiz, H., Akarsu, E., 2015. Paraoxonase and arylesterase activities in patients with papillary thyroid cancer. Scand. J. Clin. Lab. Invest. 75, 259–264. https://doi.org/10.3109/00365513.2014.1003597

Kowalska, K., Socha, E., Milnerowicz, H., 2015. Review: The role of paraoxonase in cardiovascular diseases. Ann. Clin. Lab. Sci. 45, 226–233.

Kranendonk, M., Alves, M., Antunes, P., Rueff, J., 2014. Human Sulfotransferase 1A1-Dependent Mutagenicity of 12-Hydroxy-nevirapine: The Missing Link? Chem. Res. Toxicol. 27, 1967–1971. https://doi.org/10.1021/tx5003113

Kubota, M., Nakata, R., Adachi, S., Watanabe, K.-I., Heike, T., Takeshita, Y., Shima, M., 2014. Plasma homocysteine, methionine and S-adenosylhomocysteine levels following high-dose methotrexate treatment in pediatric patients with acute lymphoblastic leukemia or Burkitt lymphoma: association with hepatotoxicity. Leuk. Lymphoma 55, 1591–1595. https://doi.org/10.3109/10428194.2013.850684

Kulka, M., 2016. A review of paraoxonase 1 properties and diagnostic applications. Pol. J. Vet. Sci. 19. https://doi.org/10.1515/pjvs-2016-0028

Kunitake, S.T., La Sala, K.J., Kane, J.P., 1985. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. J. Lipid Res. 26, 549–555.

Kunnen, S., Van Eck, M., 2012. Lecithin:cholesterol acyltransferase: old friend or foe in atherosclerosis? J. Lipid Res. 53, 1783–1799. https://doi.org/10.1194/jlr.R024513

Kunutsor, S.K., Bakker, S.J.L., James, R.W., Dullaart, R.P.F., 2016. Serum paraoxonase-1 activity and risk of incident cardiovascular disease: The PREVEND study and meta-analysis of prospective population studies. Atherosclerosis 245, 143–154. https://doi.org/10.1016/j.atherosclerosis.2015.12.021

Kuokkanen, S., Polotsky, A.J., Chosich, J., Bradford, A.P., Jasinska, A., Phang, T., Santoro, N., Appt, S.E., 2016. Corpus luteum as a novel target of weight changes that contribute to impaired female reproductive physiology and function. Syst. Biol. Reprod. Med. 62, 227–242. https://doi.org/10.3109/19396368.2016.1173743

Lakshman, M.R., Gottipati, C.S., Narasimhan, S.J., Munoz, J., Marmillot, P., Nylen, E.S., 2006. Inverse correlation of serum paraoxonase and homocysteine thiolactonase activities and antioxidant capacity of high-density lipoprotein with the severity of cardiovascular disease in persons with type 2 diabetes mellitus. Metabolism 55, 1201–1206. https://doi.org/10.1016/j.metabol.2006.06.001

Lalezari, J.P., Latiff, G.H., Brinson, C., Echevarría, J., Treviño-Pérez, S., Bogner, J.R., Thompson, M., Fourie, J., Sussmann Pena, O.A., Mendo Urbina, F.C., Martins, M., Diaconescu, I.G., Stock, D.A., Joshi, S.R., Hanna, G.J., Lataillade, M., 2015. Safety and efficacy of the HIV-1 attachment inhibitor prodrug BMS-663068 in treatment-experienced individuals: 24 week results of AI438011, a phase 2b, randomised controlled trial. Lancet HIV 2, e427–e437. https://doi.org/10.1016/S2352-3018(15)00177-0

Lam, E.W.-F., Brosens, J.J., Gomes, A.R., Koo, C.-Y., 2013. Forkhead box proteins: tuning forks for transcriptional harmony. Nat. Rev. Cancer 13, 482–495. https://doi.org/10.1038/nrc3539

Lamon-Fava, S., Ordovas, J.M., Schaefer, E.J., 1999. Estrogen increases apolipoprotein (apo) A-I secretion in hep G2 cells by modulating transcription of the apo A-I gene promoter. Arterioscler. Thromb. Vasc. Biol. 19, 2960–2965.

Lamson, M., Macgregor, T., Riska, P., Erickson, D., Maxfield, P., Rowland, L., Gigliotti, M., Robinson, P., Azzam, S., Keirns, J., 1999. Nevirapine induces both CYP3A4 and CYP2B6 metabolic pathways. Clin. Pharmacol. Ther. 65, 137–137. https://doi.org/10.1016/S0009-9236(99)80079-4

Landschulz, K.T., Pathak, R.K., Rigotti, A., Krieger, M., Hobbs, H.H., 1996. Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. J. Clin. Invest. 98, 984–995. https://doi.org/10.1172/JCI118883

Langedijk, J., Mantel-Teeuwisse, A.K., Slijkerman, D.S., Schutjens, M.-H.D.B., 2015. Drug repositioning and repurposing: terminology and definitions in literature. Drug Discov. Today 20, 1027–1034. https://doi.org/10.1016/j.drudis.2015.05.001

Lau, E., Brophy, J., Samson, L., Kakkar, F., Campbell, D.M., Yudin, M.H., Murphy, K., Seto, W., Colantonio, D., Read, S.E., Bitnun, A., 2017. Nevirapine Pharmacokinetics and Safety in Neonates Receiving Combination Antiretroviral Therapy for Prevention of Vertical HIV Transmission: JAIDS J. Acquir. Immune Defic. Syndr. 74, 493–498. https://doi.org/10.1097/QAI.0000000000001291

Law, N.C., White, M.F., Hunzicker-Dunn, M.E., 2016. G protein-coupled receptors (GPCRs) That Signal via Protein Kinase A (PKA) Cross-talk at Insulin Receptor Substrate 1 (IRS1) to Activate the phosphatidylinositol 3-kinase (PI3K)/AKT Pathway. J. Biol. Chem. 291, 27160–27169. https://doi.org/10.1074/jbc.M116.763235

Learning lessons from Pfizer's $800 million failure, 2011. . Nat. Rev. Drug Discov. 10, 163–164. https://doi.org/10.1038/nrd3401

Lee, Z., Swaby, R.F., Liang, Y., Yu, S., Liu, S., Lu, K.H., Bast, R.C., Mills, G.B., Fang, X., 2006. Lysophosphatidic Acid Is a Major Regulator of Growth-Regulated Oncogene α in Ovarian Cancer. Cancer Res. 66, 2740–2748. https://doi.org/10.1158/0008-5472.CAN-05-2947

Leman, L.J., Maryanoff, B.E., Ghadiri, M.R., 2014. Molecules That Mimic Apolipoprotein A-I: Potential Agents for Treating Atherosclerosis. J. Med. Chem. 57, 2169–2196. https://doi.org/10.1021/jm4005847

Li, H., Wang, D., Zhang, H., Kirmani, K., Zhao, Z., Steinmetz, R., Xu, Y., 2009. Lysophosphatidic acid stimulates cell migration, invasion, and colony formation as well as tumorigenesis/metastasis of mouse ovarian cancer in immunocompetent mice. Mol. Cancer Ther. 8, 1692–1701. https://doi.org/10.1158/1535-7163.MCT-08-1106

Li, X.-L., Li, J.-J., Guo, Y.-L., Zhu, C.-G., Qing, P., Wu, N.-Q., Xu, B., Gao, R.-L., 2014. The Ratio of High-Density Lipoprotein Cholesterol to Apolipoprotein A-I Predicts Myocardial Injury Following Elective Percutaneous Coronary Intervention: HDL-C/apoA-I ratio and MI after PCI. Clin. Cardiol. n/a-n/a. https://doi.org/10.1002/clc.22308

Likanonsakul, S., Rattanatham, T., Feangvad, S., Uttayamakul, S., Prasithsirikul, W., Tunthanathip, P., Nakayama, E.E., Shioda, T., 2009. HLA-Cw*04 allele associated with

nevirapine-induced rash in HIV-infected Thai patients. AIDS Res. Ther. 6, 22. https://doi.org/10.1186/1742-6405-6-22

Lin, X., Hong, S., Huang, J., Chen, Y., Chen, Y., Wu, Z., 2017. Plasma apolipoprotein A1 levels at diagnosis are independent prognostic factors in invasive ductal breast cancer. Discov. Med. 23, 247–258.

Liu, L., Zhou, L., Li, Y., Bai, W., Liu, N., Li, W., Gao, Y., Liu, Z., Han, R., 2015. High-density lipoprotein acts as an opsonin to enhance phagocytosis of group A streptococcus by U937 cells: HDL is an Opsonin. Microbiol. Immunol. 59, 419–425. https://doi.org/10.1111/1348-0421.12270

Liu, P., Cheng, H., Roberts, T.M., Zhao, J.J., 2009. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat. Rev. Drug Discov. 8, 627–644. https://doi.org/10.1038/nrd2926

Liu, Y., Burkhalter, R., Symowicz, J., Chaffin, K., Ellerbroek, S., Stack, M.S., 2012. Lysophosphatidic Acid Disrupts Junctional Integrity and Epithelial Cohesion in Ovarian Cancer Cells. J. Oncol. 2012, 1–8. https://doi.org/10.1155/2012/501492

Lokadasan, R., James, F.V., Naranayan, G., Prabhakaran, P.K., 2016. Targeted agents in epithelial ovarian cancer: review on emerging therapies and future developments. ecancermedicalscience 10. https://doi.org/10.3332/ecancer.2016.626

Lopez-Delgado, J.C., Mendiluce, R.M., Pinol, T.S., Fernández, X.P., Sanchez, L., Vicente, R.G., 2012. Urgent liver transplantation for nevirapine-induced acute liver failure: report of a case and review of the literature. Ann. Transplant. 17, 122–127.

Luo, X.-L., Zhong, G.-Z., Hu, L.-Y., Chen, J., Liang, Y., Chen, Q.-Y., Liu, Q., Rao, H.-L., Chen, K.-L., Cai, Q.-Q., 2015. Serum apolipoprotein A-I is a novel prognostic indicator for non-metastatic nasopharyngeal carcinoma. Oncotarget 6, 44037–44048. https://doi.org/10.18632/oncotarget.5823

Luoma, P.V., 2008. Cytochrome P450 and gene activation—from pharmacology to cholesterol elimination and regression of atherosclerosis. Eur. J. Clin. Pharmacol. 64, 841–850. https://doi.org/10.1007/s00228-008-0515-5

Mabuchi, S., Kuroda, H., Takahashi, R., Sasano, T., 2015. The PI3K/AKT/mTOR pathway as a therapeutic target in ovarian cancer. Gynecol. Oncol. 137, 173–179. https://doi.org/10.1016/j.ygyno.2015.02.003

Macha, S., Yong, C.-L., MacGregor, T.R., Castles, M., Quinson, A.-M., Rouyrre, N., Wilding, I., 2009. Assessment of Nevirapine Bioavailability From Targeted Sites in the Human Gastrointestinal Tract. J. Clin. Pharmacol. 49, 1417–1425. https://doi.org/10.1177/0091270009344856

Mackness, B., Beltran-Debon, R., Aragones, G., Joven, J., Camps, J., Mackness, M., 2010. Human tissue distribution of paraoxonases 1 and 2 mRNA. IUBMB Life n/a-n/a. https://doi.org/10.1002/iub.347

Mackness, B., Durrington, P.N., Abuashia, B., Boulton, A.J., Mackness, M.I., 2000. Low paraoxonase activity in type II diabetes mellitus complicated by retinopathy. Clin. Sci. Lond. Engl. 1979 98, 355–363.

Mackness, M.I., Arrol, S., Abbott, C., Durrington, P.N., 1993. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. Atherosclerosis 104, 129–135.

Maeda, S., Nakanishi, S., Yoneda, M., Awaya, T., Yamane, K., Hirano, T., Kohno, N., 2012. Associations between small dense LDL, HDL subfractions (HDL2, HDL3) and risk of atherosclerosis in Japanese-Americans. J. Atheroscler. Thromb. 19, 444–452.

Maggi, P., Bellacosa, C., Carito, V., Perilli, F., Lillo, A., Volpe, A., Trillo, G., Angiletta, D., Regina, G., Angarano, G., 2011. Cardiovascular risk factors in patients on long-term treatment with nevirapine- or efavirenz-based regimens. J. Antimicrob. Chemother. 66, 896–900. https://doi.org/10.1093/jac/dkq507

Malik, U.U., Siddiqui, I.A., Hashim, Z., Zarina, S., 2014. Measurement of serum paraoxonase activity and MDA concentrations in patients suffering with oral squamous cell carcinoma. Clin. Chim. Acta 430, 38–42. https://doi.org/10.1016/j.cca.2013.12.033

Mangiacasale, R., Pittoggi, C., Sciamanna, I., Careddu, A., Mattei, E., Lorenzini, R., Travaglini, L., Landriscina, M., Barone, C., Nervi, C., Lavia, P., Spadafora, C., 2003. Exposure of normal and transformed cells to nevirapine, a reverse transcriptase inhibitor, reduces cell growth and promotes differentiation. Oncogene 22, 2750–2761. https://doi.org/10.1038/sj.onc.1206354

Manning, B.D., 2017. Game of TOR — The Target of Rapamycin Rules Four Kingdoms. N. Engl. J. Med. 377, 1297–1299. https://doi.org/10.1056/NEJMcibr1709384

Manolescu, B.N., 2013. Paraoxonases as protective agents against N-acyl homoserine lactone - producing pathogenic microorganisms. Maedica 8, 49–52.

Marchini, J.F.M., Pinto, M.R., Novaes, G.C., Badran, A.V., Pavão, R.B., Figueiredo, G.L., Lago, I.M., Lima-Filho, M.O., Lemos, D.C., Tonani, M., Antloga, C.M., Oliveira, L., Lorenzi, J.C., Marin-Neto, J.A., Universidade de São Paulo, Brasil, Universidade de Uberaba, Brasil, Universidade de São Paulo, Brasil, Universidade de São Paulo, Brasil, 2017. Decreased platelet responsiveness to clopidogrel correlates with CYP2C19 and PON1 polymorphisms in atherosclerotic patients. Braz. J. Med. Biol. Res. 50. https://doi.org/10.1590/1414-431x20165660

Maredza, M., Bertram, M.Y., Saloojee, H., Chersich, M.F., Tollman, S.M., Hofman, K.J., 2013. Cost-effectiveness analysis of infant feeding strategies to prevent mother-to-child transmission of HIV in South Africa. Afr. J. AIDS Res. 12, 151–160. https://doi.org/10.2989/16085906.2013.863215

Marinho, A.T., Dias, C., Antunes, A., Caixas, U., Branco, T., Marques, M., Monteiro, E., Pereira, S., 2014a. Sex differences in apolipoprotein A1 and nevirapine-induced toxicity. J. Int. AIDS Soc. 17. https://doi.org/10.7448/IAS.17.4.19575

Marinho, A.T., Dias, C.G., Pinheiro, P.F., Antunes, A.M.M., Marques, M.M., Monteiro, E.C., Miranda, J.P., Pereira, S.A., 2014b. PON-1 activity as a protective player in nevirapine hepatotoxicity: Data comparison in 2D and 3D cell cultures. Toxicol. Lett. 229, S134–S135. https://doi.org/10.1016/j.toxlet.2014.06.476

Marinho, A.T., Dias, C.G., Pinheiro, P.F., Lemos, A.R., Antunes, A.M.M., Marques, M.M., Monteiro, E.C., Miranda, J.P., Pereira, S.A., 2016. Nevirapine modulation of paraoxonase-1 in the liver: An in vitro three-model approach. Eur. J. Pharm. Sci. 82, 147–153. https://doi.org/10.1016/j.ejps.2015.11.019

Marinho, A.T., Godinho, A.L.A., Novais, D.A., Antunes, A.M.M., Marques, M.M., Ramos, T., Dias, C.G., Monteiro, E.C., Pereira, S.A., 2014d. Development and validation of an HPLC-UV method for quantifying nevirapine and its main phase I metabolites in human blood. Anal. Methods 6, 1575.

Marinho, A.T., Rodrigues, P.M., Caixas, U., Antunes, A.M.M., Branco, T., Harjivan, S.G., Marques, M.M., Monteiro, E.C., Pereira, S.A., 2014c. Differences in nevirapine biotransformation as a factor for its sex-dependent dimorphic profile of adverse drug reactions. J. Antimicrob. Chemother. 69, 476–482. https://doi.org/10.1093/jac/dkt359

Marsillach, J., Mackness, B., Mackness, M., Riu, F., Beltrán, R., Joven, J., Camps, J., 2008. Immunohistochemical analysis of paraoxonases-1, 2, and 3 expression in normal mouse tissues. Free Radic. Biol. Med. 45, 146–157. https://doi.org/10.1016/j.freeradbiomed.2008.03.023

Martin, A.M., Nolan, D., James, I., Cameron, P., Keller, J., Moore, C., Phillips, E., Christiansen, F.T., Mallal, S., 2005. Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. AIDS Lond. Engl. 19, 97–99.

Martinez, L.O., Jacquet, S., Esteve, J.-P., Rolland, C., Cabezón, E., Champagne, E., Pineau, T., Georgeaud, V., Walker, J.E., Tercé, F., Collet, X., Perret, B., Barbaras, R., 2003. Ectopic

β-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. Nature 421, 75–79. https://doi.org/10.1038/nature01250

Martínez-Clemente, M., Ferré, N., González-Périz, A., López-Parra, M., Horrillo, R., Titos, E., Morán-Salvador, E., Miquel, R., Arroyo, V., Funk, C.D., Clària, J., 2010. 5-lipoxygenase deficiency reduces hepatic inflammation and tumor necrosis factor α-induced hepatocyte damage in hyperlipidemia-prone ApoE-null mice. Hepatology 51, 817–827. https://doi.org/10.1002/hep.23463

Martinez-Useros, J., Garcia-Foncillas, J., 2016. Obesity and colorectal cancer: molecular features of adipose tissue. J. Transl. Med. 14. https://doi.org/10.1186/s12967-016-0772-5

Maselli, L.M.F., Cunha, J. da, Gutierrez, E.B., Maranhão, R.C., Spada, C., Bydlowski, S.P., 2014. Human Paraoxonase-1 Activity Is Related to the Number of CD4+ T-Cells and Is Restored by Antiretroviral Therapy in HIV-1-Infected Individuals. Dis. Markers 2014, 1–7. https://doi.org/10.1155/2014/480201

Masson, D., Qatanani, M., Sberna, A.L., Xiao, R., Pais de Barros, J.P., Grober, J., Deckert, V., Athias, A., Gambert, P., Lagrost, L., Moore, D.D., Assem, M., 2008. Activation of the constitutive androstane receptor decreases HDL in wild-type and human apoA-I transgenic mice. J. Lipid Res. 49, 1682–1691. https://doi.org/10.1194/jlr.M700374-JLR200

Mathias, P.I., B'hymer, C., 2016. Mercapturic acids: recent advances in their determination by liquid chromatography/mass spectrometry and their use in toxicant metabolism studies and in occupational and environmental exposure studies. Biomarkers 21, 293–315. https://doi.org/10.3109/1354750X.2016.1141988

Maturu, V.N., Gupta, N., Singh, G., Gill, K., Sharma, Y.P., Singh, S., 2013. Serum Paraoxonase (PON1) Activity in North-West Indian Punjabi's with Acute Myocardial Infarction. Indian J. Clin. Biochem. 28, 248–254. https://doi.org/10.1007/s12291-012-0260-5

Mazerbourg, S., Kuntz, S., Grillier-Vuissoz, I., Berthe, A., Geoffroy, M., Flament, S., Bordessa, A., Boisbrun, M., 2016. Reprofiling of Troglitazone Towards More Active and Less Toxic Derivatives: A New Hope for Cancer Treatment? Curr. Top. Med. Chem. 16, 2115–2124. https://doi.org/10.2174/1568026616666160216153036

Mazur, A., 1946. An enzyme in animal tissues capable of hydrolysing the phosphorus-fluorine bond of alkyl fluorophosphates. J. Biol. Chem. 164, 271–289.

Mbuagbaw, L., Mursleen, S., Irlam, J.H., Spaulding, A.B., Rutherford, G.W., Siegfried, N., 2016. Efavirenz or nevirapine in three-drug combination therapy with two nucleoside or nucleotide-reverse transcriptase inhibitors for initial treatment of HIV infection in antiretroviral-naïve individuals, in: The Cochrane Collaboration (Ed.), Cochrane Database of Systematic Reviews. John Wiley & Sons, Ltd, Chichester, UK. https://doi.org/10.1002/14651858.CD004246.pub4

McKie, A.B., Vaughan, S., Zanini, E., Okon, I.S., Louis, L., de Sousa, C., Greene, M.I., Wang, Q., Agarwal, R., Shaposhnikov, D., Wong, J.L.C., Gungor, H., Janczar, S., El-Bahrawy, M., Lam, E.W.-F., Chayen, N.E., Gabra, H., 2012. The OPCML Tumor Suppressor Functions as a Cell Surface Repressor–Adaptor, Negatively Regulating Receptor Tyrosine Kinases in Epithelial Ovarian Cancer. Cancer Discov. 2, 156–171. https://doi.org/10.1158/2159-8290.CD-11-0256

McPherson, P.A.C., Young, I.S., McKibben, B., McEneny, J., 2006. High density lipoprotein subfractions: isolation, composition, and their duplicitous role in oxidation. J. Lipid Res. 48, 86–95. https://doi.org/10.1194/jlr.M600094-JLR200

Medrano, J., Barreiro, P., Tuma, P., Vispo, E., Labarga, P., Blanco, F., Soriano, V., 2008. Risk for immune-mediated liver reactions by nevirapine revisited. AIDS Rev. 10, 110–115.

Mehdi, M.M., Rizvi, S.I., 2012. Human Plasma Paraoxonase 1 (PON1) Arylesterase Activity During Aging: Correlation with Susceptibility of LDL Oxidation. Arch. Med. Res. 43, 438–443. https://doi.org/10.1016/j.arcmed.2012.08.012

Mei, X., Atkinson, D., 2015. Lipid-free Apolipoprotein A-I Structure: Insights into HDL Formation and Atherosclerosis Development. Arch. Med. Res. 46, 351–360. https://doi.org/10.1016/j.arcmed.2015.05.012

Melvin, J.C., Seth, D., Holmberg, L., Garmo, H., Hammar, N., Jungner, I., Walldius, G., Lambe, M., Wigertz, A., Van Hemelrijck, M., 2012. Lipid Profiles and Risk of Breast and Ovarian Cancer in the Swedish AMORIS Study. Cancer Epidemiol. Biomarkers Prev. 21, 1381–1384. https://doi.org/10.1158/1055-9965.EPI-12-0188

Meng, X., Howarth, A., Earnshaw, C.J., Jenkins, R.E., French, N.S., Back, D.J., Naisbitt, D.J., Park, B.K., 2013. Detection of Drug Bioactivation in Vivo: Mechanism of Nevirapine–Albumin Conjugate Formation in Patients. Chem. Res. Toxicol. 26, 575–583. https://doi.org/10.1021/tx4000107

Miettinen, H.E., Rayburn, H., Krieger, M., 2001. Abnormal lipoprotein metabolism and reversible female infertility in HDL receptor (SR-BI)–deficient mice. J. Clin. Invest. 108, 1717–1722. https://doi.org/10.1172/JCI200113288

Miles, R.R., Perry, W., Haas, J.V., Mosior, M.K., N'Cho, M., Wang, J.W.J., Yu, P., Calley, J., Yue, Y., Carter, Q., Han, B., Foxworthy, P., Kowala, M.C., Ryan, T.P., Solenberg, P.J., Michael, L.F., 2013. Genome-wide Screen for Modulation of Hepatic Apolipoprotein A-I (ApoA-I) Secretion. J. Biol. Chem. 288, 6386–6396. https://doi.org/10.1074/jbc.M112.410092

Millar, J.S., Cuchel, M., 2015. ApoA-I-Directed Therapies for the Management of Atherosclerosis. Curr. Atheroscler. Rep. 17. https://doi.org/10.1007/s11883-015-0539-0

Miller, K.D., Siegel, R.L., Lin, C.C., Mariotto, A.B., Kramer, J.L., Rowland, J.H., Stein, K.D., Alteri, R., Jemal, A., 2016. Cancer treatment and survivorship statistics, 2016. CA. Cancer J. Clin. 66, 271–289. https://doi.org/10.3322/caac.21349

Mills, G.B., Moolenaar, W.H., 2003. The emerging role of lysophosphatidic acid in cancer. Nat. Rev. Cancer 3, 582–591. https://doi.org/10.1038/nrc1143

Min, G., Kim, H., Bae, Y., Petz, L., Kemper, J.K., 2002. Inhibitory Cross-talk between Estrogen Receptor (ER) and Constitutively Activated Androstane Receptor (CAR): CAR INHIBITS ER-MEDIATED SIGNALING PATHWAY BY SQUELCHING p160 COACTIVATORS. J. Biol. Chem. 277, 34626–34633. https://doi.org/10.1074/jbc.M205239200

Miranda, J.P., Leite, S.B., Muller-Vieira, U., Rodrigues, A., Carrondo, M.J.T., Alves, P.M., 2009. Towards an Extended Functional Hepatocyte *In Vitro* Culture. Tissue Eng. Part C Methods 15, 157–167. https://doi.org/10.1089/ten.tec.2008.0352

Mishra, V.K., Palgunachari, M.N., McPherson, D.T., Anantharamaiah, G.M., 2013. Lipid complex of apolipoprotein A-I mimetic peptide 4F is a novel platform for paraoxonase-1 binding and enhancing its activity and stability. Biochem. Biophys. Res. Commun. 430, 975–980. https://doi.org/10.1016/j.bbrc.2012.11.128

Modoni, S., Landriscina, M., Fabiano, A., Fersini, A., Urbano, N., Ambrosi, A., Cignarelli, M., 2007. Reinduction of Cell Differentiation and ^{131}I Uptake in a Poorly Differentiated Thyroid Tumor in Response to the Reverse Transcriptase (RT) Inhibitor Nevirapine. Cancer Biother. Radiopharm. 22, 289–295. https://doi.org/10.1089/cbr.2006.316

Moritz, A., Li, Y., Guo, A., Villen, J., Wang, Y., MacNeill, J., Kornhauser, J., Sprott, K., Zhou, J., Possemato, A., Ren, J.M., Hornbeck, P., Cantley, L.C., Gygi, S.P., Rush, J., Comb, M.J., 2010. Akt-RSK-S6 Kinase Signaling Networks Activated by Oncogenic Receptor Tyrosine Kinases. Sci. Signal. 3, ra64-ra64. https://doi.org/10.1126/scisignal.2000998

Mukherjee, R., Locke, K.T., Miao, B., Meyers, D., Monshizadegan, H., Zhang, R., Search, D., Grimm, D., Flynn, M., O'Malley, K.M., Zhang, L., Li, J., Shi, Y., Kennedy, L.J., Blanar, M., Cheng, P.T., Tino, J., Srivastava, R.A., 2008. Novel Peroxisome Proliferator-Activated Receptor Agonists Lower Low-Density Lipoprotein and Triglycerides, Raise High-Density Lipoprotein, and Synergistically Increase Cholesterol Excretion with a Liver X Receptor Agonist. J. Pharmacol. Exp. Ther. 327, 716–726. https://doi.org/10.1124/jpet.108.143271

Murphy, A.J., Westerterp, M., Yvan-Charvet, L., Tall, A.R., 2012. Anti-atherogenic mechanisms of high density lipoprotein: Effects on myeloid cells. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1821, 513–521. https://doi.org/10.1016/j.bbalip.2011.08.003

Nagar, S., Walther, S., Blanchard, R.L., 2006. Sulfotransferase (SULT) 1A1 Polymorphic Variants *1, *2, and *3 Are Associated with Altered Enzymatic Activity, Cellular Phenotype, and Protein Degradation. Mol. Pharmacol. 69, 2084–2092. https://doi.org/10.1124/mol.105.019240

Naik, A., Belič, A., Zanger, U.M., Rozman, D., 2013. Molecular Interactions between NAFLD and Xenobiotic Metabolism. Front. Genet. 4. https://doi.org/10.3389/fgene.2013.00002

Nakamura, K., Sawada, K., Kinose, Y., Yoshimura, A., Toda, A., Nakatsuka, E., Hashimoto, K., Mabuchi, S., Morishige, K., Kurachi, H., Lengyel, E., Kimura, T., 2017. Exosomes Promote Ovarian Cancer Cell Invasion through Transfer of CD44 to Peritoneal Mesothelial Cells. Mol. Cancer Res. 15, 78–92. https://doi.org/10.1158/1541-7786.MCR-16-0191

Navab, M., Reddy, S.T., Anantharamaiah, G.M., Imaizumi, S., Hough, G., Hama, S., Fogelman, A.M., 2011. Intestine may be a major site of action for the apoA-I mimetic peptide 4F whether administered subcutaneously or orally. J. Lipid Res. 52, 1200–1210. https://doi.org/10.1194/jlr.M013144

Nestal de Moraes, G., Bella, L., Zona, S., Burton, M.J., Lam, E.W.-F., 2016. Insights into a Critical Role of the FOXO3a-FOXM1 Axis in DNA Damage Response and Genotoxic Drug Resistance. Curr. Drug Targets 17, 164–177.

Ng, W., Lobach, A.R.M., Zhu, X., Chen, X., Liu, F., Metushi, I.G., Sharma, A., Li, J., Cai, P., Ip, J., Novalen, M., Popovic, M., Zhang, X., Tanino, T., Nakagawa, T., Li, Y., Uetrecht, J., 2012. Animal Models of Idiosyncratic Drug Reactions, in: Advances in Pharmacology. Elsevier, pp. 81–135. https://doi.org/10.1016/B978-0-12-398339-8.00003-3

Nguyen, D., Nickel, M., Mizuguchi, C., Saito, H., Lund-Katz, S., Phillips, M.C., 2013. Interactions of Apolipoprotein A-I with High-Density Lipoprotein Particles. Biochemistry (Mosc.) 52, 1963–1972. https://doi.org/10.1021/bi400032y

Nguyen, S.D., Hung, N.D., Cheon-Ho, P., Ree, K.M., Dai-Eun, S., 2009. Oxidative inactivation of lactonase activity of purified human paraoxonase 1 (PON1). Biochim. Biophys. Acta BBA - Gen. Subj. 1790, 155–160. https://doi.org/10.1016/j.bbagen.2008.11.009

Nicholls, S.J., Brewer, H.B., Kastelein, J.J.P., Krueger, K.A., Wang, M.-D., Shao, M., Hu, B., McErlean, E., Nissen, S.E., 2011a. Effects of the CETP Inhibitor Evacetrapib Administered as Monotherapy or in Combination With Statins on HDL and LDL Cholesterol: A Randomized Controlled Trial. JAMA 306. https://doi.org/10.1001/jama.2011.1649

Nicholls, S.J., Gordon, A., Johansson, J., Wolski, K., Ballantyne, C.M., Kastelein, J.J.P., Taylor, A., Borgman, M., Nissen, S.E., 2011b. Efficacy and Safety of a Novel Oral Inducer of Apolipoprotein A-I Synthesis in Statin-Treated Patients With Stable Coronary Artery Disease. J. Am. Coll. Cardiol. 57, 1111–1119. https://doi.org/10.1016/j.jacc.2010.11.015

Nicholls, S.J., Puri, R., Wolski, K., Ballantyne, C.M., Barter, P.J., Brewer, H.B., Kastelein, J.J.P., Hu, B., Uno, K., Kataoka, Y., Herrman, J.-P.R., Merkely, B., Borgman, M., Nissen, S.E., 2016. Effect of the BET Protein Inhibitor, RVX-208, on Progression of Coronary Atherosclerosis: Results of the Phase 2b, Randomized, Double-Blind, Multicenter, ASSURE Trial. Am. J. Cardiovasc. Drugs 16, 55–65. https://doi.org/10.1007/s40256-015-0146-z

Niedernhofer, L.J., Daniels, J.S., Rouzer, C.A., Greene, R.E., Marnett, L.J., 2003. Malondialdehyde, a Product of Lipid Peroxidation, Is Mutagenic in Human Cells. J. Biol. Chem. 278, 31426–31433. https://doi.org/10.1074/jbc.M212549200

Niesor, E., 2015. Will Lipidation of ApoA1 through Interaction with ABCA1 at the Intestinal Level Affect the Protective Functions of HDL? Biology 4, 17–38. https://doi.org/10.3390/biology4010017

Nissen, S.E., Nicholls, S.J., Wolski, K., Howey, D.C., McErlean, E., Wang, M.-D., Gomez, E.V., Russo, J.M., 2007. Effects of a Potent and Selective PPAR-α Agonist in Patients With Atherogenic Dyslipidemia or Hypercholesterolemia: Two Randomized Controlled Trials. JAMA 297, 1362. https://doi.org/10.1001/jama.297.12.1362

Nofer, J.-R., Kehrel, B., Fobker, M., Levkau, B., Assmann, G., von Eckardstein, A., 2002. HDL and arteriosclerosis: beyond reverse cholesterol transport. Atherosclerosis 161, 1–16.

Nolen, B.M., Lokshin, A.E., 2013. Biomarker Testing for Ovarian Cancer: Clinical Utility of Multiplex Assays. Mol. Diagn. Ther. 17, 139–146. https://doi.org/10.1007/s40291-013-0027-6

Oda, K., Ikeda, Y., Kawana, K., Osuga, Y., Fujii, T., 2015. mTOR Signaling in Endometrial Cancer: From a Molecular and Therapeutic Point of View. Curr. Obstet. Gynecol. Rep. 4, 1–10. https://doi.org/10.1007/s13669-014-0103-x

Ogunro, P.S., Idogun, E.S., Ogungbamigbe, T.O., Ajala, M.O., Olowu, O.A., 2008. Serum concentration of acute phase protein and lipid profile in HIV-1 seropositive patients and its relationship to the progression of the disease. Niger. Postgrad. Med. J. 15, 219–224.

Ordovas, J.M., Corella, D., Cupples, L.A., Demissie, S., Kelleher, A., Coltell, O., Wilson, P.W.F., Schaefer, E.J., Tucker, K., 2002. Polyunsaturated fatty acids modulate the effects of the APOA1 G-A polymorphism on HDL-cholesterol concentrations in a sex-specific manner: the Framingham Study. Am. J. Clin. Nutr. 75, 38–46.

Oreagba, I.A., Usman, S.O., Olayemi, S.O., Oshikoya, K.A., Opanuga, O., Adeyemo, T.A., Lesi, O.A., Dodoo, A.N., Akanmu, A.S., 2014. Pharmacoepidemiology of antiretroviral drugs in a teaching hospital in Lagos, Nigeria. Ghana Med. J. 48, 194–203.

Oronsky, B., Ray, C.M., Spira, A.I., Trepel, J.B., Carter, C.A., Cottrill, H.M., 2017. A brief review of the management of platinum-resistant–platinum-refractory ovarian cancer. Med. Oncol. 34. https://doi.org/10.1007/s12032-017-0960-z

Ottevanger, P.B., 2017. Ovarian cancer stem cells more questions than answers. Semin. Cancer Biol. 44, 67–71. https://doi.org/10.1016/j.semcancer.2017.04.009

Oversteegen, L., Shah, M., Rovini, H., 2007. HIV combination products. Nat. Rev. Drug Discov. 6, 951–952. https://doi.org/10.1038/nrd2448

Oyesanya, R.A., Greenbaum, S., Dang, D., Lee, Z., Mukherjee, A., Wu, J., Dent, P., Fang, X., 2010. Differential requirement of the epidermal growth factor receptor for G protein-mediated activation of transcription factors by lysophosphatidic acid. Mol. Cancer 9, 8. https://doi.org/10.1186/1476-4598-9-8

Paemanee, A., Sornjai, W., Kittisenachai, S., Sirinonthanawech, N., Roytrakul, S., Wongtrakul, J., Smith, D.R., 2017. Nevirapine induced mitochondrial dysfunction in HepG2 cells. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-09321-y

Paik, S., 2016. Fatal Nevirapine-Induced Toxic Epidermal Necrolysis in a HIV Infected Patient. J. Clin. Diagn. Res. https://doi.org/10.7860/JCDR/2016/16360.7415

Palmirotta, R., Silvestris, E., D'Oronzo, S., Cardascia, A., Silvestris, F., 2017. Ovarian cancer: Novel molecular aspects for clinical assessment. Crit. Rev. Oncol. Hematol. 117, 12–29. https://doi.org/10.1016/j.critrevonc.2017.06.007

Pastryk, J.E., Rusek, M., Bełtowski, J., 2016. Effects of antiretroviral treatment on paraoxonase 1 (PON1) activity in rats. Chem. Biol. Interact. 259, 407–412. https://doi.org/10.1016/j.cbi.2016.06.031

Pawar, M.P., 2015. Nevirapine: Most Common Cause of Cutaneous Adverse Drug Reactions in an Outpatient Department of a Tertiary Care Hospital. J. Clin. Diagn. Res. https://doi.org/10.7860/JCDR/2015/13672.6768

Peng, D.-J., Wang, J., Zhou, J.-Y., Wu, G.S., 2010. Role of the Akt/mTOR survival pathway in cisplatin resistance in ovarian cancer cells. Biochem. Biophys. Res. Commun. 394, 600–605. https://doi.org/10.1016/j.bbrc.2010.03.029

Pereira, S.A., Batuca, J.R., Caixas, U., Branco, T., Delgado-Alves, J., Germano, I., Lampreia, F., Monteiro, E.C., 2009. Effect of efavirenz on high-density lipoprotein antioxidant properties in HIV-infected patients. Br. J. Clin. Pharmacol. 68, 891–897. https://doi.org/10.1111/j.1365-2125.2009.03535.x

Pereira, S.A., Branco, T., Corte-Real, R.M., Germano, I., Lampreia, F., Caixas, U., Monteiro, E.C., 2006. Long-term and concentration-dependent beneficial effect of efavirenz on HDL-cholesterol in HIV-infected patients. Br. J. Clin. Pharmacol. 61, 601–604. https://doi.org/10.1111/j.1365-2125.2006.02619.x

Perła-Kaján, J., Jakubowski, H., 2012. Paraoxonase 1 and homocysteine metabolism. Amino Acids 43, 1405–1417. https://doi.org/10.1007/s00726-012-1321-z

Picaud, S., Wells, C., Felletar, I., Brotherton, D., Martin, S., Savitsky, P., Diez-Dacal, B., Philpott, M., Bountra, C., Lingard, H., Fedorov, O., Muller, S., Brennan, P.E., Knapp, S., Filippakopoulos, P., 2013. RVX-208, an inhibitor of BET transcriptional regulators with selectivity for the second bromodomain. Proc. Natl. Acad. Sci. 110, 19754–19759. https://doi.org/10.1073/pnas.1310658110

Pinheiro, P.F., Marinho, A.T., Antunes, A.M.M., Marques, M.M., Pereira, S.A., Miranda, J.P., 2015. Sex differences in hepatic and intestinal contributions to nevirapine biotransformation in rats. Chem. Biol. Interact. 233, 115–121. https://doi.org/10.1016/j.cbi.2015.03.024

Pinheiro, P.F., Pereira, S.A., Harjivan, S.G., Martins, I.L., Marinho, A.T., Cipriano, M., Jacob, C.C., Oliveira, N.G., Castro, M.F., Marques, M.M., Antunes, A.M.M., Miranda, J.P., 2017. Hepatocyte spheroids as a competent in vitro system for drug biotransformation studies: nevirapine as a bioactivation case study. Arch. Toxicol. 91, 1199–1211. https://doi.org/10.1007/s00204-016-1792-x

Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review. Eur. J. Med. Chem. 97, 55–74. https://doi.org/10.1016/j.ejmech.2015.04.040

Pittoggi, C., Martis, G., Mastrangeli, G., Mastrangeli, B., Spadafora, C., 2008. In vitro evidence for a new therapeutic approach in renal cell carcinoma. Int. Braz. J. Urol. 34, 492–502. https://doi.org/10.1590/S1677-55382008000400012

Podzamczer, D., Andrade-Villanueva, J., Clotet, B., Taylor, S., Rockstroh, J., Reiss, P., Domingo, P., Gellermann, H., de Rossi, L., Cairns, V., Soriano, V., 2011. Lipid profiles for nevirapine vs. atazanavir/ritonavir, both combined with tenofovir disoproxil fumarate and emtricitabine over 48 weeks, in treatment-naïve HIV-1-infected patients (the ARTEN study): NVP vs. ATZ/r in naïve HIV-infected patients: ARTEN lipid profiles. HIV Med. 12, 374–382. https://doi.org/10.1111/j.1468-1293.2011.00917.x

Podzamczer, D., Fredy Rojas, J., Neves, I., Ferrer, E., Leal, M., Gorgolas, M., Jose, C.M., Gatell, J.M., Correia Abreu, R., Curto, J., Domingo, P., Pilar, B.M., Rozas, N., 2014. Effectiveness and tolerability of abacavir-lamivudine-nevirapine (ABC/3TC/NVP) in a multicentre cohort of HIV-infected, ARV-naïve patients. J. Int. AIDS Soc. 17. https://doi.org/10.7448/IAS.17.4.19773

Podzamczer, D., Tiraboschi, J.M., Mallolas, J., Curto, J., Cárdenes, M.A., Casas, E., Castro, A., Echevarría, S., Leal, M., Lopez Bernaldo de Quirós, J.C., Moreno, S., Puig, T., Ribera, E., Villalonga, C., Gómez-Sirvent, J.L., García-Henarejos, J.A., Lopez-Aldeguer, J., Barrufet, P., Force, L., Santos, I., Sanz, J., 2012. Long-term benefits of nevirapine-containing regimens: multicenter study with 506 patients, followed-up a median of 9 years. Curr. HIV Res. 10, 513–520.

Pollard, R.B., Robinson, P., Dransfield, K., 1998. Safety profile of nevirapine, a nonnucleoside reverse transcriptase inhibitor for the treatment of human immunodeficiency virus infection. Clin. Ther. 20, 1071–1092.

Ponce-Ruiz, N., Murillo-González, F.E., Rojas-García, A.E., Mackness, M., Bernal-Hernández, Y.Y., Barrón-Vivanco, B.S., González-Arias, C.A., Medina-Díaz, I.M., 2017. Transcriptional regulation of human Paraoxonase 1 by nuclear receptors. Chem. Biol. Interact. 268, 77–84. https://doi.org/10.1016/j.cbi.2017.02.005

Popovic, M., Caswell, J.L., Mannargudi, B., Shenton, J.M., Uetrecht, J.P., 2006. Study of the Sequence of Events Involved in Nevirapine-Induced Skin Rash in Brown Norway Rats. Chem. Res. Toxicol. 19, 1205–1214. https://doi.org/10.1021/tx0601152

Popovic, M., Shenton, J.M., Chen, J., Baban, A., Tharmanathan, T., Mannargudi, B., Abdulla, D., Uetrecht, J.P., 2010. Nevirapine Hypersensitivity, in: Uetrecht, J. (Ed.), Adverse Drug Reactions. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 437–451. https://doi.org/10.1007/978-3-642-00663-0_15

Pyne, N.J., Waters, C., Moughal, N.A., Sambi, B.S., Pyne, S., 2003. Receptor tyrosine kinase–GPCR signal complexes. Biochem. Soc. Trans. 31, 1220–1225. https://doi.org/10.1042/bst0311220

Qin, X., Chen, Q., Sun, C., Wang, C., Peng, Q., Xie, L., Liu, Y., Li, S., 2013. High-throughput screening of tumor metastatic-related differential glycoprotein in hepatocellular carcinoma by iTRAQ combines lectin-related techniques. Med. Oncol. 30. https://doi.org/10.1007/s12032-012-0420-8

Rainwater, D.L., Rutherford, S., Dyer, T.D., Rainwater, E.D., Cole, S.A., VandeBerg, J.L., Almasy, L., Blangero, J., MacCluer, J.W., Mahaney, M.C., 2009. Determinants of variation in human serum paraoxonase activity. Heredity 102, 147–154. https://doi.org/10.1038/hdy.2008.110

Reddy, K.B., Nabha, S.M., Atanaskova, N., 2003. Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev. 22, 395–403.

Rhee, E.-J., Byrne, C.D., Sung, K.-C., 2017. The HDL cholesterol/apolipoprotein A-I ratio: an indicator of cardiovascular disease. Curr. Opin. Endocrinol. Diabetes Obes. 24, 148–153. https://doi.org/10.1097/MED.0000000000000315

Riaz, A., Huang, Y., Johansson, S., 2016. G-Protein-Coupled Lysophosphatidic Acid Receptors and Their Regulation of AKT Signaling. Int. J. Mol. Sci. 17, 215. https://doi.org/10.3390/ijms17020215

Riska, Joseph, D.P., Dinallo, R.M., Davidson, W.C., Keirns, J.J., Hattox, S.E., 1999a. Biotransformation of nevirapine, a non-nucleoside HIV-1 reverse transcriptase inhibitor, in mice, rats, rabbits, dogs, monkeys, and chimpanzees. Drug Metab. Dispos. Biol. Fate Chem. 27, 1434–1447.

Riska, Lamson, M., MacGregor, T., Sabo, J., Hattox, S., Pav, J., Keirns, J., 1999b. Disposition and biotransformation of the antiretroviral drug nevirapine in humans. Drug Metab. Dispos. Biol. Fate Chem. 27, 895–901.

Rižner, T.L., 2016. Discovery of biomarkers for endometrial cancer: current status and prospects. Expert Rev. Mol. Diagn. 16, 1315–1336. https://doi.org/10.1080/14737159.2016.1258302

Rizov, M., Andreeva, P., Dimova, I., 2017. Molecular regulation and role of angiogenesis in reproduction. Taiwan. J. Obstet. Gynecol. 56, 127–132. https://doi.org/10.1016/j.tjog.2016.06.019

Rizzo, M., Otvos, J., Nikolic, D., Montalto, G., Toth, P.P., Banach, M., 2014. Subfractions and Subpopulations of HDL: An Update. Curr. Med. Chem. 21, 2881–2891. https://doi.org/10.2174/09298673216661404141034 55

Rogue, A., Spire, C., Brun, M., Claude, N., Guillouzo, A., 2010. Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and Human Liver. PPAR Res. 2010, 1–16. https://doi.org/10.1155/2010/325183

Rohrer, L., Hersberger, M., von Eckardstein, A., 2004. High density lipoproteins in the intersection of diabetes mellitus, inflammation and cardiovascular disease. Curr. Opin. Lipidol. 15, 269–278.

Rokx, C., Verbon, A., Rijnders, B.J.A., 2015. Short Communication: Lipids and Cardiovascular Risk After Switching HIV-1 Patients on Nevirapine and Emtricitabine/Tenofovir-DF to Rilpivirine/Emtricitabine/Tenofovir-DF. AIDS Res. Hum. Retroviruses 31, 363–367. https://doi.org/10.1089/aid.2014.0278

Rosenson, R.S., Brewer, H.B., Ansell, B.J., Barter, P., Chapman, M.J., Heinecke, J.W., Kontush, A., Tall, A.R., Webb, N.R., 2015. Dysfunctional HDL and atherosclerotic cardiovascular disease. Nat. Rev. Cardiol. 13, 48–60. https://doi.org/10.1038/nrcardio.2015.124

Ruiz, L., Negredo, E., Domingo, P., Paredes, R., Francia, E., Balagué, M., Gel, S., Bonjoch, A., Fumaz, C.R., Johnston, S., Romeu, J., Lange, J., Clotet, B., Spanish Lipodystrophy Group, 2001. Antiretroviral treatment simplification with nevirapine in protease inhibitor-experienced patients with hiv-associated lipodystrophy: 1-year prospective follow-up of a multicenter, randomized, controlled study. J. Acquir. Immune Defic. Syndr. 1999 27, 229–236.

Rysz-Górzyńska, M., Banach, M., 2016. Subfractions of high-density lipoprotein (HDL) and dysfunctional HDL in chronic kidney disease patients. Arch. Med. Sci. 4, 844–849. https://doi.org/10.5114/aoms.2016.60971

Sako, A., Kitayama, J., Shida, D., Suzuki, R., Sakai, T., Ohta, H., Nagawa, H., 2006. Lysophosphatidic Acid (LPA)-Induced Vascular Endothelial Growth Factor (VEGF) by Mesothelial Cells and Quantification of Host-Derived VEGF in Malignant Ascites. J. Surg. Res. 130, 94–101. https://doi.org/10.1016/j.jss.2005.08.007

Sankatsing, R.R., Wit, F.W., Pakker, N., Vyankandondera, J., Mmiro, F., Okong, P., Kastelein, J.J., Lange, J.M., Stroes, E.S., Reiss, P., 2007. Effects of Nevirapine, Compared with Lamivudine, on Lipids and Lipoproteins in HIV-1–Uninfected Newborns: The Stopping Infection from Mother-to-Child via Breast-Feeding in Africa Lipid Substudy. J. Infect. Dis. 196, 15–22. https://doi.org/10.1086/518248

Santos, J.M., Camões, S.P., Filipe, E., Cipriano, M., Barcia, R.N., Filipe, M., Teixeira, M., Simões, S., Gaspar, M., Mosqueira, D., Nascimento, D.S., Pinto-do-Ó, P., Cruz, P., Cruz, H., Castro, M., Miranda, J.P., 2015. Three-dimensional spheroid cell culture of umbilical cord tissue-derived mesenchymal stromal cells leads to enhanced paracrine induction of wound healing. Stem Cell Res. Ther. 6. https://doi.org/10.1186/s13287-015-0082-5

Sasano, T., Mabuchi, S., Kuroda, H., Kawano, M., Matsumoto, Y., Takahashi, R., Hisamatsu, T., Sawada, K., Hashimoto, K., Isobe, A., Testa, J.R., Kimura, T., 2015. Preclinical Efficacy for AKT Targeting in Clear Cell Carcinoma of the Ovary. Mol. Cancer Res. 13, 795–806. https://doi.org/10.1158/1541-7786.MCR-14-0314

Satta, N., Pagano, S., Montecucco, F., Gencer, B., Mach, F., Kaiser, L., Calmy, A., Vuilleumier, N., Aubert, V., Barth, J., Battegay, M., Bernasconi, E., Böni, J., Bucher, H.C., Burton-Jeangros, C., Calmy, A., Cavassini, M., Egger, M., Elzi, L., Fehr, J., Fellay, J., Francioli, P., Furrer, H., Fux, C.A., Gorgievski, M., Günthard, H., Haerry, D., Hasse, B., Hirsch, H.H., Hirschel, B., Hösli, I., Kahlert, C., Kaiser, L., Keiser, O., Kind, C., Klimkait, T., Kovari, H., Ledergerber, B., Martinetti, G., Martinez de Tejada, B., Metzner, K., Müller, N., Nadal, D., Pantaleo, G., Rauch, A., Regenass, S., Rickenbach, M., Rudin, C., Schmid, P., Schultze, D., Schöni-Affolter, F., Schüpbach, J., Speck, R., Taffé, P., Tarr, P., Telenti, A., Trkola, A., Vernazza, P., Weber, R., Yerly, S., 2017. Anti-apolipoprotein A-1 autoantibodies are associated with immunodeficiency and systemic inflammation in HIV patients. J. Infect. https://doi.org/10.1016/j.jinf.2017.11.008

Saunders, J.A., Rogers, L.C., Klomsiri, C., Poole, L.B., Daniel, L.W., 2010. Reactive oxygen species mediate lysophosphatidic acid induced signaling in ovarian cancer cells. Free Radic. Biol. Med. 49, 2058–2067. https://doi.org/10.1016/j.freeradbiomed.2010.10.663

Schrader, C., Ernst, I.M.A., Sinnecker, H., Soukup, S.T., Kulling, S.E., Rimbach, G., 2012. Genistein as a potential inducer of the anti-atherogenic enzyme paraoxonase-1: studies in cultured hepatocytes *in vitro* and in rat liver *in vivo*. J. Cell. Mol. Med. 16, 2331–2341. https://doi.org/10.1111/j.1582-4934.2012.01542.x

Schreier, L.E., Berg, G.A., Basilio, F.M., Lopez, G.I., Etkin, A.E., Wikinski, R.L., 1999. Lipoprotein alterations, abdominal fat distribution and breast cancer. Biochem. Mol. Biol. Int. 47, 681–690.

Sciamanna, I., Landriscina, M., Pittoggi, C., Quirino, M., Mearelli, C., Beraldi, R., Mattei, E., Serafino, A., Cassano, A., Sinibaldi-Vallebona, P., Garaci, E., Barone, C., Spadafora, C., 2005. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene 24, 3923–3931. https://doi.org/10.1038/sj.onc.1208562

Sehitogulları, A., Aslan, M., Sayır, F., Kahraman, A., Demir, H., 2014. Serum paraoxonase-1 enzyme activities and oxidative stress levels in patients with esophageal squamous cell carcinoma. Redox Rep. 19, 199–205. https://doi.org/10.1179/1351000214Y.0000000091

Sena, C.M., Pereira, A.M., Seiça, R., 2013. Endothelial dysfunction — A major mediator of diabetic vascular disease. Biochim. Biophys. Acta BBA - Mol. Basis Dis. 1832, 2216–2231. https://doi.org/10.1016/j.bbadis.2013.08.006

Sharma, A.M., Novalen, M., Tanino, T., Uetrecht, J.P., 2013. 12-OH-Nevirapine Sulfate, Formed in the Skin, Is Responsible for Nevirapine-Induced Skin Rash. Chem. Res. Toxicol. 26, 817–827. https://doi.org/10.1021/tx400098z

Sharma, G.S., Kumar, T., Dar, T.A., Singh, L.R., 2015. Protein N-homocysteinylation: From cellular toxicity to neurodegeneration. Biochim. Biophys. Acta BBA - Gen. Subj. 1850, 2239–2245. https://doi.org/10.1016/j.bbagen.2015.08.013

Shenton, J.M., Popovic, M., Chen, J., Masson, M.J., Uetrecht, J.P., 2005. Evidence of an Immune-Mediated Mechanism for an Idiosyncratic Nevirapine-Induced Reaction in the Female Brown Norway Rat. Chem. Res. Toxicol. 18, 1799–1813. https://doi.org/10.1021/tx0501132

Shepard, H.M., Brdlik, C.M., Schreiber, H., 2008. Signal integration: a framework for understanding the efficacy of therapeutics targeting the human EGFR family. J. Clin. Invest. 118, 3574–3581. https://doi.org/10.1172/JCI36049

Shiratsuki, S., Hara, T., Munakata, Y., Shirasuna, K., Kuwayama, T., Iwata, H., 2016. Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells. Mol. Cell. Endocrinol. 437, 75–85. https://doi.org/10.1016/j.mce.2016.08.010

Sikora, M., Rae, J., Johnson, M., Desta, Z., 2010. Efavirenz directly modulates the oestrogen receptor and induces breast cancer cell growth: Efavirenz modulates the oestrogen receptor. HIV Med. 11, 603–607. https://doi.org/10.1111/j.1468-1293.2010.00831.x

Simpson, E.R., Rochelle, D.B., Carr, B.R., MacDonald, P.C., 1980. PLASMA LIPOPROTEINS IN FOLLICULAR FLUID OF HUMAN OVARIES. J. Clin. Endocrinol. Metab. 51, 1469–1471. https://doi.org/10.1210/jcem-51-6-1469

Sinibaldi-Vallebona, P., Lavia, P., Garaci, E., Spadafora, C., 2006. A role for endogenous reverse transcriptase in tumorigenesis and as a target in differentiating cancer therapy. Genes. Chromosomes Cancer 45, 1–10. https://doi.org/10.1002/gcc.20266

Sirniö, P., Väyrynen, J.P., Klintrup, K., Mäkelä, J., Mäkinen, M.J., Karttunen, T.J., Tuomisto, A., 2017. Decreased serum apolipoprotein A1 levels are associated with poor survival and systemic inflammatory response in colorectal cancer. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-05415-9

Slatter, D.A., Paul, R.G., Murray, M., Bailey, A.J., 1999. Reactions of lipid-derived malondialdehyde with collagen. J. Biol. Chem. 274, 19661–19669.

Smith, C.V., Jones, D.P., Guenthner, T.M., Lash, L.H., Lauterburg, B.H., 1996. Compartmentation of Glutathione: Implications for the Study of Toxicity and Disease. Toxicol. Appl. Pharmacol. 140, 1–12. https://doi.org/10.1006/taap.1996.0191

Smith, P.F., DiCenzo, R., Morse, G.D., 2001. Clinical Pharmacokinetics of Non-Nucleoside Reverse Transcriptase Inhibitors: Clin. Pharmacokinet. 40, 893–905. https://doi.org/10.2165/00003088-200140120-00002

Smolle, E., Taucher, V., Pichler, M., Petru, E., Lax, S., Haybaeck, J., 2013. Targeting Signaling Pathways in Epithelial Ovarian Cancer. Int. J. Mol. Sci. 14, 9536–9555. https://doi.org/10.3390/ijms14059536

Soran, H., Schofield, J.D., Liu, Y., Durrington, P.N., 2015. How HDL protects LDL against atherogenic modification: paraoxonase 1 and other dramatis personae. Curr. Opin. Lipidol. 26, 247–256. https://doi.org/10.1097/MOL.0000000000000194

Soriano, V., Arastéh, K., Migrone, H., Lutz, T., Opravil, M., Andrade-Villanueva, J., Antunes, F., Di Perri, G., Podzamczer, D., Taylor, S., Domingo, P., Gellermann, H., de Rossi, L., ARTEN investigators, 2011. Nevirapine versus atazanavir/ritonavir, each combined with tenofovir disoproxil fumarate/emtricitabine, in antiretroviral-naive HIV-1 patients: the ARTEN Trial. Antivir. Ther. 16, 339–348. https://doi.org/10.3851/IMP1745

Spang, J.E., Bertrand, S., Westera, G., Patt, J.T., Schubiger, P.A., Bertrand, D., 2000. Chemical modification of epibatidine causes a switch from agonist to antagonist and modifies its selectivity for neuronal nicotinic acetylcholine receptors. Chem. Biol. 7, 545–555.

Srinivas, R.V., Rui, Z., Owens, R.J., Compans, R.W., Venkatachalapathi, Y.V., Gupta, K.B., Srinivas, S.K., Anantharamaiah, G.M., Segrest, J.P., 1991. Inhibition of virus-induced cell fusion by apolipoprotein A-I and its amphipathic peptide analogs. J. Cell. Biochem. 45, 224–237. https://doi.org/10.1002/jcb.240450214

Sriraman, V., Sinha, M., Richards, J.S., 2010. Progesterone Receptor-Induced Gene Expression in Primary Mouse Granulosa Cell Cultures1. Biol. Reprod. 82, 402–412. https://doi.org/10.1095/biolreprod.109.077610

Srivastava, A., Lian, L.-Y., Maggs, J.L., Chaponda, M., Pirmohamed, M., Williams, D.P., Park, B.K., 2010. Quantifying the Metabolic Activation of Nevirapine in Patients by Integrated Applications of NMR and Mass Spectrometries. Drug Metab. Dispos. 38, 122–132. https://doi.org/10.1124/dmd.109.028688

Stavnes, H.T., Nymoen, D.A., Hetland Falkenthal, T.E., Kaern, J., Trope, C.G., Davidson, B., 2014. APOA1 mRNA Expression in Ovarian Serous Carcinoma Effusions Is a Marker of Longer Survival. Am. J. Clin. Pathol. 142, 51–57. https://doi.org/10.1309/AJCPD8NBSHXRXQL7

Steeg, P.S., 2016. Targeting metastasis. Nat. Rev. Cancer 16, 201–218. https://doi.org/10.1038/nrc.2016.25

Stevens, J., Lyall, H., 2014. Mother to child transmission of HIV: What works and how much is enough? J. Infect. 69, S56–S62. https://doi.org/10.1016/j.jinf.2014.07.018

Stouffer, R.L., Bishop, C.V., Bogan, R.L., Xu, F., Hennebold, J.D., 2013. Endocrine and local control of the primate corpus luteum. Reprod. Biol. 13, 259–271. https://doi.org/10.1016/j.repbio.2013.08.002

Strehlau, R., Coovadia, A., Abrams, E.J., Martens, L., Arpadi, S., Meyers, T., Kuhn, L., 2012. Lipid Profiles in Young HIV-Infected Children Initiating and Changing Antiretroviral Therapy: JAIDS J. Acquir. Immune Defic. Syndr. 60, 369–376. https://doi.org/10.1097/QAI.0b013e318243760b

Stronach, E., Cheraghchi, A., Chen, M., Gabra, H., 2011. Targeting the AKT Pathway in Ovarian Cancer, in: Kaye, S., Brown, R., Gabra, H., Gore, M. (Eds.), Emerging Therapeutic Targets in Ovarian Cancer. Springer-Verlag, New York, pp. 73–94.

Su, F., Kozak, K.R., Imaizumi, S., Gao, F., Amneus, M.W., Grijalva, V., Ng, C., Wagner, A., Hough, G., Farias-Eisner, G., Anantharamaiah, G.M., Van Lenten, B.J., Navab, M., Fogelman, A.M., Reddy, S.T., Farias-Eisner, R., 2010. Apolipoprotein A-I (apoA-I) and apoA-I mimetic peptides inhibit tumor development in a mouse model of ovarian cancer. Proc. Natl. Acad. Sci. 107, 19997–20002. https://doi.org/10.1073/pnas.1009010107

Suiko, M., Kurogi, K., Hashiguchi, T., Sakakibara, Y., Liu, M.-C., 2017. Updated perspectives on the cytosolic sulfotransferases (SULTs) and SULT-mediated sulfation. Biosci. Biotechnol. Biochem. 81, 63–72. https://doi.org/10.1080/09168451.2016.1222266

Sullivan, D., Olsson, A.G., Scott, R., Kim, J.B., Xue, A., Gebski, V., Wasserman, S.M., Stein, E.A., 2012. Effect of a Monoclonal Antibody to PCSK9 on Low-Density Lipoprotein Cholesterol Levels in Statin-Intolerant Patients: The GAUSS Randomized Trial. JAMA 308, 2497. https://doi.org/10.1001/jama.2012.25790

Sullivan, L.B., Chandel, N.S., 2014. Mitochondrial reactive oxygen species and cancer. Cancer Metab. 2, 17. https://doi.org/10.1186/2049-3002-2-17

Sulzmaier, F.J., Jean, C., Schlaepfer, D.D., 2014. FAK in cancer: mechanistic findings and clinical applications. Nat. Rev. Cancer 14, 598–610. https://doi.org/10.1038/nrc3792

Sun, T., Hu, J., Yin, Z., Xu, Z., Zhang, L., Fan, L., Zhuo, Y., Wang, C., 2017. Low serum paraoxonase1 activity levels predict coronary artery disease severity. Oncotarget. https://doi.org/10.18632/oncotarget.14305

Sung, K.-C., Ryu, S., Wild, S.H., Byrne, C.D., 2015. An increased high-density lipoprotein cholesterol/apolipoprotein A-I ratio is associated with increased cardiovascular and all-cause mortality. Heart 101, 553–558. https://doi.org/10.1136/heartjnl-2014-306784

Sutherland, W.H.F., Manning, P.J., de Jong, S.A., Allum, A.R., Jones, S.D., Williams, S.M., 2001. Hormone-replacement therapy increases serum paraoxonase arylesterase activity in diabetic postmenopausal women. Metabolism 50, 319–324. https://doi.org/10.1053/meta.2001.20201

Suzuki, Y., Umemura, T., Ishii, Y., Hibi, D., Inoue, T., Jin, M., Sakai, H., Kodama, Y., Nohmi, T., Yanai, T., Nishikawa, A., Ogawa, K., 2012. Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice. Mutat. Res. Toxicol. Environ. Mutagen. 749, 23–28. https://doi.org/10.1016/j.mrgentox.2012.07.002

Svärd, J., Blanco, F., Nevin, D., Fayne, D., Mulcahy, F., Hennessy, M., Spiers, J.P., 2014. Differential interactions of antiretroviral agents with LXR, ER and GR nuclear receptors: potential contributing factors to adverse events: Novel ARV drug-nuclear receptor interactions. Br. J. Pharmacol. 171, 480–497. https://doi.org/10.1111/bph.12480

Swedish Human Protein Atlas Project, n.d. Swedish Human Protein Atlas Project. [WWW Document]. PON1 - Tissue Atlas. URL http://www.proteinatlas.org/ENSG00000005421-PON1/tissue (accessed 5.23.17).

Sys, G.M.L., Lapeire, L., Stevens, N., Favoreel, H., Forsyth, R., Bracke, M., De Wever, O., 2013. The In ovo CAM-assay as a Xenograft Model for Sarcoma. J. Vis. Exp. https://doi.org/10.3791/50522

Tada, N., Sakamoto, T., Kagami, A., Mochizuki, K., Kurosaka, K., 1993. Antimicrobial activity of lipoprotein particles containing apolipoprotein Al. Mol. Cell. Biochem. 119, 171–178.

Taiwo, B.O., 2006. Nevirapine toxicity. Int. J. STD AIDS 17, 364–370. https://doi.org/10.1258/095646206777323346

Takano, M., Kikuchi, Y., Asakawa, T., Goto, T., Kita, T., Kudoh, K., Kigawa, J., Sakuragi, N., Sakamoto, M., Sugiyama, T., Yaegashi, N., Tsuda, H., Seto, H., Shiwa, M., 2010. Identification of potential serum markers for endometrial cancer using protein expression profiling. J. Cancer Res. Clin. Oncol. 136, 475–481. https://doi.org/10.1007/s00432-009-0680-7

The AIM-HIGH Investigators, 2011. Niacin in Patients with Low HDL Cholesterol Levels Receiving Intensive Statin Therapy. N. Engl. J. Med. 365, 2255–2267. https://doi.org/10.1056/NEJMoa1107579

The Emerging Risk Factors Collaboration*, 2009. Major Lipids, Apolipoproteins, and Risk of Vascular Disease. JAMA 302, 1993. https://doi.org/10.1001/jama.2009.1619

The HPS2-THRIVE Collaborative Group, 2014. Effects of Extended-Release Niacin with Laropiprant in High-Risk Patients. N. Engl. J. Med. 371, 203–212. https://doi.org/10.1056/NEJMoa1300955

Thein, P., Kalinec, G.M., Park, C., Kalinec, F., 2014. In vitro assessment of antiretroviral drugs demonstrates potential for ototoxicity. Hear. Res. 310, 27–35. https://doi.org/10.1016/j.heares.2014.01.005

Thibault, B., Castells, M., Delord, J.-P., Couderc, B., 2014. Ovarian cancer microenvironment: implications for cancer dissemination and chemoresistance acquisition. Cancer Metastasis Rev. 33, 17–39. https://doi.org/10.1007/s10555-013-9456-2

Thomas, M., Burk, O., Klumpp, B., Kandel, B.A., Damm, G., Weiss, T.S., Klein, K., Schwab, M., Zanger, U.M., 2013. Direct Transcriptional Regulation of Human Hepatic Cytochrome P450 3A4 (CYP3A4) by Peroxisome Proliferator-Activated Receptor Alpha (PPAR). Mol. Pharmacol. 83, 709–718. https://doi.org/10.1124/mol.112.082503

Tian, L., Li, C., Liu, Y., Chen, Y., Fu, M., 2014. The Value and Distribution of High-Density Lipoprotein Subclass in Patients with Acute Coronary Syndrome. PLoS ONE 9, e85114. https://doi.org/10.1371/journal.pone.0085114

Tohyama, J., Billheimer, J.T., Fuki, I.V., Rothblat, G.H., Rader, D.J., Millar, J.S., 2009. Effects of nevirapine and efavirenz on HDL cholesterol levels and reverse cholesterol transport in mice. Atherosclerosis 204, 418–423. https://doi.org/10.1016/j.atherosclerosis.2008.09.016

Trinh, X.B., Tjalma, W.A.A., Vermeulen, P.B., Van den Eynden, G., Van der Auwera, I., Van Laere, S.J., Helleman, J., Berns, E.M.J.J., Dirix, L.Y., van Dam, P.A., 2009. The VEGF pathway and the AKT/mTOR/p70S6K1 signalling pathway in human epithelial ovarian cancer. Br. J. Cancer 100, 971–978. https://doi.org/10.1038/sj.bjc.6604921

Tseng, Y.-T., Yang, C.-J., Chang, S.-Y., Lin, S.-W., Tsai, M.-S., Liu, W.-C., Wu, P.-Y., Su, Y.-C., Luo, Y.-Z., Yang, S.-P., Hung, C.-C., Chang, S.-C., 2014. Incidence and risk factors of skin rashes and hepatotoxicity in HIV-infected patients receiving nevirapine-containing combination antiretroviral therapy in Taiwan. Int. J. Infect. Dis. 29, 12–17. https://doi.org/10.1016/j.ijid.2014.08.012

Tsoi, C., Falany, C.N., Morgenstern, R., Swedmark, S., 2001. Identification of a new subfamily of sulphotransferases: cloning and characterization of canine SULT1D1. Biochem. J. 356, 891–897.

Tuteja, S., Rader, D.J., 2014. High-Density Lipoproteins in the Prevention of Cardiovascular Disease: Changing the Paradigm. Clin. Pharmacol. Ther. 96, 48–56. https://doi.org/10.1038/clpt.2014.79

Valiyaveettil, M., Alamneh, Y.A., Doctor, B.P., Nambiar, M.P., 2012. Crossroads in the evaluation of paraoxonase 1 for protection against nerve agent and organophosphate toxicity. Toxicol. Lett. 210, 87–94. https://doi.org/10.1016/j.toxlet.2012.01.013

van der Valk, M., Kastelein, J.J., Murphy, R.L., van Leth, F., Katlama, C., Horban, A., Glesby, M., Behrens, G., Clotet, B., Stellato, R.K., Molhuizen, H.O., Reiss, P., Atlantic Study Team, 2001. Nevirapine-containing antiretroviral therapy in HIV-1 infected patients results in an anti-atherogenic lipid profile. AIDS Lond. Engl. 15, 2407–2414.

Van Lenten, B.J., Wagner, A.C., Anantharamaiah, G.M., Navab, M., Reddy, S.T., Buga, G.M., Fogelman, A.M., 2009. Apolipoprotein A-I Mimetic Peptides. Curr Atheroscler Rep 11, 52–57.

Van Lenten, B.J., Wagner, A.C., Jung, C.-L., Ruchala, P., Waring, A.J., Lehrer, R.I., Watson, A.D., Hama, S., Navab, M., Anantharamaiah, G.M., Fogelman, A.M., 2008. Anti-inflammatory apoA-I-mimetic peptides bind oxidized lipids with much higher affinity than human apoA-I. J. Lipid Res. 49, 2302–2311. https://doi.org/10.1194/jlr.M800075-JLR200

van Leth, F., Phanuphak, P., Stroes, E., Gazzard, B., Cahn, P., Raffi, F., Wood, R., Bloch, M., Katlama, C., Kastelein, J.J.P., Schechter, M., Murphy, R.L., Horban, A., Hall, D.B., Lange, J.M.A., Reiss, P., 2004. Nevirapine and Efavirenz Elicit Different Changes in Lipid Profiles in Antiretroviral- Therapy-Naive Patients Infected with HIV-1. PLoS Med. 1, e19. https://doi.org/10.1371/journal.pmed.0010019

Van Linthout, S., Frias, M., Singh, N., De Geest, B., 2015. Therapeutic Potential of HDL in Cardioprotection and Tissue Repair, in: von Eckardstein, A., Kardassis, D. (Eds.), High Density Lipoproteins. Springer International Publishing, Cham, pp. 527–565. https://doi.org/10.1007/978-3-319-09665-0_17

Vaz, F.M., Ferdinandusse, S., 2017. Bile acid analysis in human disorders of bile acid biosynthesis. Mol. Aspects Med. https://doi.org/10.1016/j.mam.2017.03.003

Vedhachalam, C., Duong, P.T., Nickel, M., Nguyen, D., Dhanasekaran, P., Saito, H., Rothblat, G.H., Lund-Katz, S., Phillips, M.C., 2007. Mechanism of ATP-binding Cassette Transporter A1-mediated Cellular Lipid Efflux to Apolipoprotein A-I and Formation of High Density Lipoprotein Particles. J. Biol. Chem. 282, 25123–25130. https://doi.org/10.1074/jbc.M704590200

Venkatachalam, K.V., Akita, H., Strott, C.A., 1998. Molecular cloning, expression, and characterization of human bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase and its functional domains. J. Biol. Chem. 273, 19311–19320.

Vinci, M., Box, C., Eccles, S.A., 2015. Three-Dimensional (3D) Tumor Spheroid Invasion Assay. J. Vis. Exp. https://doi.org/10.3791/52686

von Otte, S., 2005. Follicular Fluid High Density Lipoprotein-associated Sphingosine 1-Phosphate Is a Novel Mediator of Ovarian Angiogenesis. J. Biol. Chem. 281, 5398–5405. https://doi.org/10.1074/jbc.M508759200

Walter, S., Weinschenk, T., Stenzl, A., Zdrojowy, R., Pluzanska, A., Szczylik, C., Staehler, M., Brugger, W., Dietrich, P.-Y., Mendrzyk, R., Hilf, N., Schoor, O., Fritsche, J., Mahr, A., Maurer, D., Vass, V., Trautwein, C., Lewandrowski, P., Flohr, C., Pohla, H., Stanczak, J.J., Bronte, V., Mandruzzato, S., Biedermann, T., Pawelec, G., Derhovanessian, E., Yamagishi, H., Miki, T., Hongo, F., Takaha, N., Hirakawa, K., Tanaka, H., Stevanovic, S., Frisch, J., Mayer-Mokler, A., Kirner, A., Rammensee, H.-G., Reinhardt, C., Singh-Jasuja, H., 2012. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat. Med. 18, 1254–1261. https://doi.org/10.1038/nm.2883

Wang, X., Dai, S., Zhang, Z., Liu, L., Wang, J., Xiao, X., He, D., Liu, B., 2009. Characterization of apolipoprotein A-I as a potential biomarker for cholangiocarcinoma. Eur. J. Cancer Care (Engl.) 18, 625–635. https://doi.org/10.1111/j.1365-2354.2008.00965.x

Wang, Y., Meng, C., Wei, Q., Shi, F., Mao, D., 2015. Expression and regulation of scavenger receptor class B type 1 in the rat ovary and uterus during the estrous cycle. Acta Histochem. 117, 297–304. https://doi.org/10.1016/j.acthis.2015.03.007

Ward, D., Slim, J., 2013. Considerations on the New Nevirapine: Switching Patients from Twice-Daily to Once-Daily. J. Int. Assoc. Provid. AIDS Care JIAPAC 12, 154–156. https://doi.org/10.1177/2325957412473648

Ward, J.D., Dhanasekaran, D.N., 2012. LPA Stimulates the Phosphorylation of p130Cas via G i2 in Ovarian Cancer Cells. Genes Cancer 3, 578–591. https://doi.org/10.1177/1947601913475360

Webber, K., Friedlander, M., 2017. Chemotherapy for epithelial ovarian, fallopian tube and primary peritoneal cancer. Best Pract. Res. Clin. Obstet. Gynaecol. 41, 126–138. https://doi.org/10.1016/j.bpobgyn.2016.11.004

Wegdam, W., Argmann, C.A., Kramer, G., Vissers, J.P., Buist, M.R., Kenter, G.G., Aerts, J.M.F.G., Meijer, D., Moerland, P.D., 2014. Label-Free LC-MSe in Tissue and Serum Reveals Protein Networks Underlying Differences between Benign and Malignant Serous Ovarian Tumors. PLoS ONE 9, e108046. https://doi.org/10.1371/journal.pone.0108046

Wen, B., Chen, Y., Fitch, W.L., 2009. Metabolic Activation of Nevirapine in Human Liver Microsomes: Dehydrogenation and Inactivation of Cytochrome P450 3A4. Drug Metab. Dispos. 37, 1557–1562. https://doi.org/10.1124/dmd.108.024851

Wen, X., Donepudi, A.C., Thomas, P.E., Slitt, A.L., King, R.S., Aleksunes, L.M., 2013. Regulation of Hepatic Phase II Metabolism in Pregnant Mice. J. Pharmacol. Exp. Ther. 344, 244–252. https://doi.org/10.1124/jpet.112.199034

Wilhelm, A.J., Zabalawi, M., Owen, J.S., Shah, D., Grayson, J.M., Major, A.S., Bhat, S., Gibbs, D.P., Thomas, M.J., Sorci-Thomas, M.G., 2010. Apolipoprotein A-I Modulates Regulatory T Cells in Autoimmune LDLr-/-, ApoA-I-/- Mice. J. Biol. Chem. 285, 36158–36169. https://doi.org/10.1074/jbc.M110.134130

Wilkening, S., Stahl, F., Bader, A., 2003. COMPARISON OF PRIMARY HUMAN HEPATOCYTES AND HEPATOMA CELL LINE HEPG2 WITH REGARD TO THEIR BIOTRANSFORMATION PROPERTIES. Drug Metab. Dispos. 31, 1035–1042. https://doi.org/10.1124/dmd.31.8.1035

Woods, D.C., White, Y.A.R., Dau, C., Johnson, A.L., 2011. TLR4 activates NF-κB in human ovarian granulosa tumor cells. Biochem. Biophys. Res. Commun. 409, 675–680. https://doi.org/10.1016/j.bbrc.2011.05.063

World Health Organization, 2016. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach.

Woudberg, N.J., Goedecke, J.H., Blackhurst, D., Frias, M., James, R., Opie, L.H., Lecour, S., 2016. Association between ethnicity and obesity with high-density lipoprotein (HDL) function and subclass distribution. Lipids Health Dis. 15. https://doi.org/10.1186/s12944-016-0257-9

Wu, B., Kulkarni, K., Basu, S., Zhang, S., Hu, M., 2011. First-Pass Metabolism via UDP-Glucuronosyltransferase: a Barrier to Oral Bioavailability of Phenolics. J. Pharm. Sci. 100, 3655–3681. https://doi.org/10.1002/jps.22568

Wu, S., Gao, X., Yang, S., Meng, M., Yang, X., Ge, B., 2015. The role of endoplasmic reticulum stress in endothelial dysfunction induced by homocysteine thiolactone. Fundam. Clin. Pharmacol. 29, 252–259. https://doi.org/10.1111/fcp.12101

Wu, W., Kocarek, T.A., Runge-Morris, M., 2001. Sex-dependent regulation by dexamethasone of murine hydroxysteroid sulfotransferase gene expression. Toxicol. Lett. 119, 235–246.

Xie, Q., Zhao, S.-P., Li, F., 2010. D-4F, an Apolipoprotein A-I Mimetic Peptide, Promotes Cholesterol Efflux from Macrophages via ATP-Binding Cassette Transporter A1. Tohoku J. Exp. Med. 220, 223–228. https://doi.org/10.1620/tjem.220.223

Xu, Y., Fang, X.J., Casey, G., Mills, G.B., 1995. Lysophospholipids activate ovarian and breast cancer cells. Biochem. J. 309 (Pt 3), 933–940.

Yanai, H., Javitt, N., Higashi, Y., Fuda, H., Strott, C., 2004. Expression of Cholesterol Sulfotransferase (SULT2B1b) in Human Platelets. Circulation 109, 92–96. https://doi.org/10.1161/01.CIR.0000108925.95658.8D

Yeh, C., Cheng, C.-C., Lin, H.-C., Luo, T.-Y., Chang, J., Ho, A.-S., 2016. Pravastatin inhibits tumor growth through elevating the levels of apolipoprotein A1. Adv. Dig. Med. 3, 3–10. https://doi.org/10.1016/j.aidm.2015.03.003

Yi, F., Guo, J., Dabbagh, D., Spear, M., He, S., Kehn-Hall, K., Fontenot, J., Yin, Y., Bibian, M., Park, C.M., Zheng, K., Park, H., Soloveva, V., Gharaibeh, D., Retterer, C., Zamani, R., Pitt, M.L., Naughton, J., Jiang, Y., Shang, H., Hakami, R.M., Ling, B., Young, J.A.T., Bavari, S., Xu, X., Feng, Y., Wu, Y., 2017. Discovery of Novel Small Molecule Inhibitors of LIM Domain Kinase for Inhibiting HIV-1. J. Virol. JVI.02418-16. https://doi.org/10.1128/JVI.02418-16

Ying, R., Yuan, Y., Qin, Y.-F., Tian, D., Feng, L., Guo, Z.-G., Sun, Y.-X., Li, M.-X., 2013. The combination of L-4F and simvastatin stimulate cholesterol efflux and related proteins expressions to reduce atherosclerotic lesions in apoE knockout mice. Lipids Health Dis. 12, 180. https://doi.org/10.1186/1476-511X-12-180

Yu, B., Wang, S., Peng, D., Zhao, S., 2010. HDL and immunomodulation: an emerging role of HDL against atherosclerosis. Immunol. Cell Biol. 88, 285–290. https://doi.org/10.1038/icb.2009.112

Yuan, J., Guo, S., Hall, D., Cammett, A.M., Jayadev, S., Distel, M., Storfer, S., Huang, Z., Mootsikapun, P., Ruxrungtham, K., Podzamczer, D., Haas, D.W., 2011. Toxicogenomics of nevirapine-associated cutaneous and hepatic adverse events among populations of African, Asian, and European descent: AIDS 25, 1271–1280. https://doi.org/10.1097/QAD.0b013e32834779df

Yuhanna, I.S., Zhu, Y., Cox, B.E., Hahner, L.D., Osborne-Lawrence, S., Lu, P., Marcel, Y.L., Anderson, R.G., Mendelsohn, M.E., Hobbs, H.H., Shaul, P.W., 2001. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. Nat. Med. 7, 853–857. https://doi.org/10.1038/89986

Zamanian-Daryoush, M., DiDonato, J.A., 2015. Apolipoprotein A-I and Cancer. Front. Pharmacol. 6. https://doi.org/10.3389/fphar.2015.00265

Zamanian-Daryoush, M., Lindner, D., Tallant, T.C., Wang, Z., Buffa, J., Klipfell, E., Parker, Y., Hatala, D., Parsons-Wingerter, P., Rayman, P., Yusufishaq, M.S.S., Fisher, E.A., Smith, J.D., Finke, J., DiDonato, J.A., Hazen, S.L., 2013. The Cardioprotective Protein Apolipoprotein A1 Promotes Potent Anti-tumorigenic Effects. J. Biol. Chem. 288, 21237–21252. https://doi.org/10.1074/jbc.M113.468967

Zhang, M., Xiong, H., Fang, L., Lu, W., Wu, X., Huang, Z.-S., Wang, Y.-Q., Cai, Z.-M., Wu, S., 2015. Paraoxonase 1 (PON1) Q192R Gene Polymorphism and Cancer Risk: A Meta-Analysis Based on 30 Publications. Asian Pac. J. Cancer Prev. APJCP 16, 4457–4463.

Zhao, F., Siu, M.K.Y., Jiang, L., Tam, K.F., Ngan, H.Y.S., Le, X.F., Wong, O.G.W., Wong, E.S.Y., Gomes, A.R., Bella, L., Khongkow, P., Lam, E.W.-F., Cheung, A.N.Y., 2014. Overexpression of Forkhead Box Protein M1 (FOXM1) in Ovarian Cancer Correlates with Poor Patient Survival and Contributes to Paclitaxel Resistance. PLoS ONE 9, e113478. https://doi.org/10.1371/journal.pone.0113478

Zhou, M., McFarland-Mancini, M.M., Funk, H.M., Husseinzadeh, N., Mounajjed, T., Drew, A.F., 2009. Toll-like receptor expression in normal ovary and ovarian tumors. Cancer Immunol. Immunother. 58, 1375–1385. https://doi.org/10.1007/s00262-008-0650-y

Žitňanová, I., Rakovan, M., Paduchová, Z., Dvořáková, M., Andrezálová, L., Muchová, J., Šimko, M., Waczulíková, I., Ďuračková, Z., 2011. Oxidative stress in women with perimenopausal symptoms: Menopause 18, 1249–1255. https://doi.org/10.1097/gme.0b013e318224fa3d